

NPT in serious trouble

Once again, a review conference of the Nuclear Non-Proliferation Treaty has ended in disarray. But this time, the consequences could be serious.

JUST before dawn last Saturday, the quinquennial review conference of the Non-Proliferation Treaty (NPT) at Geneva reached the dispiriting conclusion that it could not agree on a final statement embodying the conclusions reached in the preceding two weeks. While the immediate cause of failure was the insistence of the Mexican delegation that the signatory nuclear powers should commit themselves to a comprehensive nuclear test-ban treaty by the time of the next review conference in five years, the underlying trouble seems to have been the general ill-temper of the non-nuclear signatories.

Yet is this not the year in which the nuclear signatories will be able to make good their promise to "achieve at the earliest possible date" an end to the "nuclear arms race", "nuclear disarmament" and "general and complete disarmament under strict and effective international control"? Since the 1985 review conference, after all, the Soviet Union and the United States have signed and ratified the treaty on nuclear missiles of intermediate range (INF). Will there not now also be the START treaty to limit Soviet and US strategic weapons, as well as an agreement on conventional forces in Europe?

That was the outlook when this year began, as it was at the superpower summit in Malta earlier in the year. But the prospects have faded as the months have passed. Europeans may have been cheered by the good things that have happened in the past few months in Central Europe, but there is no reason why NPT signatories elsewhere should be buoyed up by that good fortune. Instead, they have the trouble in the Gulf to worry them.

The Gulf, or the Middle East in general, is not irrelevant to the NPT. Israel (not a signatory of the treaty) is assumed to be a nuclear power on the strength of its technical capability, its secretiveness about its activities at the Dimona site in the Negev and the calculation (in which there is some force) that nuclear weapons might effectively be used, in the last resort, to give pause to a seemingly successful conventional attack. The arguments used since 1945 by acknowledged nuclear powers could well have persuaded other Middle East states to follow suit. So, naturally, there has from time to time been talk of building an "Islamic bomb", usually centred on the alleged ambitions of Pakistan (which, like India, is not a signatory of the treaty). If more is not done, there will be one some day.

Yet Iraq is dutifully a signatory, as are Israel's other

neighbours such as Egypt, Jordan and Syria. So (further away) are Iran and Kuwait. The only notable exception is Saudi Arabia. Yet Iraq was accused by Israel in 1981 (when it bombed out of action the research reactor supplied by France) of nursing nuclear ambitions. Circumstantial evidence since then, as well as the evident fascination of the Iraqi leadership with elaborate and unconventional weapons systems (not to mention poison gas), suggests that the accusation may have been correct.

That does not, of course, excuse the illegal bombing of the reactor; it would have been more effective if evidence of Iraq's intentions had been used to ensure that the International Atomic Energy Agency (IAEA) carried out a "special" inspection as allowed under the terms of its agreements with signatory states. That might even have forestalled some of the present trouble.

For the long haul, and for the long-term future of the NPT, persisting uncertainties in the Middle East are a literally frightening example to other regions of the world. Whether Israel and one or more of its potential adversaries are nuclear powers may be less important than the perceptions of states elsewhere. Some of them may reflect that if it is now the case that, for a nation to walk tall in a violent world, its adversaries must be made to regard it as putatively nuclear, they had better take appropriate steps themselves. In short, the example of the Middle East and, more generally, the likelihood that the waning of superpower equipartition of influence will allow regional conflicts to become more common, has eroded previous restraints on military nuclear development. The pressure in that direction is increased by the continuing self-exclusion from the NPT of China and France, both acknowledged nuclear powers, and by the likelihood that the years ahead will put a premium on civil nuclear power generation.

In the circumstances, Mexico's protest at Geneva was not merely unrealistic but counter-productive. If Mexico's objective was to strengthen the non-proliferation regime, it chose an odd way to that end. To demand that governments should agree to agree, and on the spot, is to deny the realities of international relations, however much they have improved. Only a little reflection would have shown that a comprehensive test-ban would be a curious animal without the adherence of France and China, which the signatories of the NPT are not in a position to command.



Moreover, the experience of the past few years has shown that major arms control agreements depend on the undistracted eagerness of powerful leaders for a deal. Without the enthusiasm of Mr Mikhail Gorbachev, there would not have been an INF treaty. He may yet be able to pull a START treaty and a conventional weapons agreement out of the hat, despite the obstacles erected by the Soviet military (over submarine-launched cruise missiles and aircraft respectively), but the signs are not as good as they were at the beginning of the year. For Mexico to have scorned the compromise offered at Geneva last week, which would have committed all the signatories to some kind of activity, was principled to the point of perversity.

So what is to be done? The next conference is that at which the NPT will lapse unless its signatories agree to renew it. That would be a tragic outcome, especially because of the constructive proposals for operating and interpreting the NPT agreed last week. These include a proposed undertaking that nuclear suppliers should provide nuclear materials only to countries accepting international safeguards on all their nuclear plants and even a "negative assurance" that nuclear states will not use nuclear weapons against non-nuclear countries.

Luckily, the treaty as it stands allows a procedure for amendments to be proposed by any signatory and one by which a third of the signatories can require an *ad hoc* conference to consider them. The obvious next step is that some well-intentioned government should offer as an amendment the conference statement as it might have been. When tempers have cooled a little, even Mexico might consider that a valuable way of making amends. Otherwise, some other state should take the initiative. To let the fate of nuclear proliferation rest on governments' separate interpretation of last week's aborted review conference is too dangerous. Then, Mexico will have made a debating point and ruined the most durable of all the arms control agreements. □

Bad luck insurance

Confidentiality will complicate attempts to sequence the human genome in ways that should be faced.

REPRESENTATIVE John Conyers (see page 221) is not alone in worrying about the use that will be made of information relating to the nucleotide sequences of genes carried by individuals. So, too, are the managers of the Human Genome Project, which will be a prolific source of information about the general and also the detailed architecture of the human genome. With good planning and much hard work, there will be a comprehensive listing of all functioning human genes and information about the ways in which they can vary, either harmlessly or otherwise, from one individual to another. No doubt there will develop alongside the project a flourishing trade in the diagnosis of specified genetic defects (already possible in the growing number of cases in which suitable

probes have been identified). The issue is whether information relating to individuals should be disclosed to outside parties — employers, insurance companies or even intended spouses, for example.

The simple answer is that there should be no disclosure. The rules that bind physicians not to disclose information about their patients to outsiders, however vivid outsiders' interests, should apply equally strictly to those who sequence genes. Conyers's bill, whatever happens to it in the US Congress, will serve the useful purpose of emphasizing that point, but it may well be redundant even as the law now stands. Yet those who are cheering on the bill in the belief that its enactment would avoid some awkward social problems are probably mistaken.

Insurance is an important case. Insurance companies already require those applying for life insurance to answer questions about their medical backgrounds and their occupations, requiring honest answers on pain of forfeiture of benefits (not to mention premiums). If, as is probable, the Human Genome Project makes possible the more certain diagnosis of, say, the genetic determinants of heart disease, what will there be to prevent an insurance company from requiring that applicants should undergo the appropriate diagnostic test? In principle, that would not differ from the present requirement that some applicants for insurance should be examined medically before a policy is written. The practice could not be forbidden in the case of genetic information yet continued with older technology without becoming unconstitutional.

Nor would it be equitable. Just as non-smokers would have cause for complaint if insurance companies were required to offer the same terms to cigarette-smokers as to themselves, people free of specified genetic defects would rightly complain if those carrying defective and potentially damaging genes were knowingly offered insurance on standard terms. So, at some stage, genetic sequencing information is bound to become an issue. Moreover, mere reticence will not avoid the obvious untoward consequences, but will rather lose the social benefits that will arise if and when people have a better understanding of the human genetic constitution and its variations (including an appreciation that the overwhelming proportion are harmless variations).

That is why blanket legislation will not defend people against insurance companies. But people (and the new technology) need defending by a requirement that it should not be incumbent on a person to commission an investigation of his or her genetic constitution before applying for insurance, and that, once insured, he or she should not be disqualified because unwelcome information afterwards comes to light. Then what should happen to those who, perhaps because of some family investigation, are already known to be carrying an uninsurable gene? Why should people be penalized for bad luck is the question asked? The remedy, not the answer, is that congenital bad luck is a communal responsibility — a principle only slowly being accepted in the United States. □

Financial realities hit US science budget

■ Grants may be cut by 50 per cent

■ Space missions face cancellation

Washington

THE optimism of last January's White House budget request for science has finally given way before the realities of the gigantic government deficit and economic slowdown. The 15 per cent boost in major scientific research areas announced nine months ago is now on its way down, reaching 12 per cent in the latest budget figures available this week. But as budget negotiators make a last-ditch effort to bring the US deficit under control, science budgets could still be cut by some 30 per cent across the board.

Although congressional support of science has been stronger this year than many had expected, the estimated \$150,000 million national deficit (excluding the still unknown — and probably massive — cost of rescuing the crumbling savings and loan industry) leaves little room for magnanimity in budget negotiations.

All federal agencies have been asked to make provisional plans for a 32.4 per cent reduction, the amount required to bring the 1991 budget deficit under the ceiling prescribed by the Gramm–Rudman–Hollings deficit-reduction act. Unless Congress changes the law or reaches some other compromise in the next week, Gramm–Rudman takes effect on 1 October. Federal agencies were forced to absorb Gramm–Rudman cuts for almost two months last year while congressional negotiators hammered out a budget agreement that met the deficit ceilings.

Assuming that Congress moves no more quickly this year (it seems likely that legislators will wait until after the November elections to make unpopular budget cuts), federal science agencies are preparing to make draconian funding reductions on the first of the month. The National Science Foundation (NSF) has warned funding recipients that existing grants will be cut by 50 per cent from 1 October, to allow some new grants to be awarded. "Unless there's some miracle, a 50 per cent cut seems likely", says NSF budget officer Joel Whitter. A year-long Gramm–Rudman sequestration (admittedly unlikely) would mean 21,000 fewer scientists and students supported by NSF grants.

At the National Institutes of Health (NIH), budget officials are also preparing to make difficult compromises. Latest figures suggest that existing grants would

be reduced by around 30 per cent in dollar amount in the event of sequestration. The number of new and competing grants will be reduced by about 70 per cent while Gramm–Rudman cuts are in effect. If Congress delays in reaching a budget compromise, "we are talking in terms of a significant reduction in existing grants to allow for new ones", says one NIH budget officer.

Department of Energy (DOE) research facilities are likely to be another victim of austerity. Protracted sequestration would

1991 US SCIENCE BUDGET (\$ million)

	1990 Budget	1991 Request	House*	Senate*
NIH	7,277	7,623	8,318	8,003
NSF	2,080	2,383	2,337	2,360
NASA	12,221	15,125	14,296	13,450
DOE (R&D)	3,283	3,359	3,976	4,019

* Budgets approved by congressional subcommittees.

stop construction of the Continuous Electron Beam Accelerator Facility and halt the beginning of the Superconducting Supercollider and the Relativistic Heavy Ion Collider. Existing accelerator facilities such as Fermilab and the Stanford Linear Accelerator Centre would find their operating budgets cut by at least half.

Congressional priorities

Although the budget negotiations make bottom-line figures necessarily soft, the appropriations process is still an accurate gauge of Congress' science priorities, and a good indicator of cuts to come. Last week, appropriations subcommittees passed the Senate's versions of the budgets for the National Aeronautics and Space Administration (NASA), NSF and NIH. The bills join the already completed House measures and wait only for the conclusion of the budget negotiations before their differences can be reconciled in conference and final appropriations voted on.

The biggest surprises lie mostly at NASA, where the Senate joined the House in eliminating almost all funding for the Moon/Mars initiative, for which the president had requested \$300 million.

The Senate subcommittee also cut \$864 million (35 per cent) from the space station, more than four times the House cut and a significant blow to the programme.

Although the space station may be able to weather the loss, some smaller projects face outright cancellation. The CRAF/Cassini cometary exploration probes lost

\$50 million from their \$148 million request. Because work must begin soon on the spacecraft if they are to be ready in time for a mid-1990s launch window (when the intended comets will be within reach), even a one-year funding delay could cause NASA to abandon parts of the project. As the European Space Agency has already committed itself to the Cassini mission, CRAF appears to be the odd man out. "If the end result of the \$50 million cut is that one of them has to be dropped, it will be CRAF", says NASA project manager Henry Brenton.

The Senate also shifted funding within the Earth Observation System programme from large satellites to smaller ones, and restored funding for an existing search for extraterrestrial life that the House bill had derisively deleted. The crippled Hubble space telescope was given \$30 million in extra funding to help speed corrective measures.

Among the federal science agencies, NSF fared best last week, as the Senate appropriations subcommittee approved a 14 per cent increase to \$2,360 million, a figure that lies close to the presidential request. Like the House, however, the Senate eliminated funds for the Laser

Interferometer Gravitational Wave Detector and emphasized education increases over research.

No crisis at NIH?

In last week's NIH bill, the Senate differed from the House in granting the full administration request for the genome project (\$108 million; the House had cut that to \$66 million [see *Nature* 346, 309; 26 July 1990]). With the exception of AIDS funding, which the Senate reduced by some \$300 million, overall NIH funding in both bills represents a 10 per cent increase over last year.

Like the House bill, the Senate report is expected to include controversial language instructing NIH to reform their grant process. Among the directives, the House bill calls for NIH to increase the number of grants they issue to 6,000 (compared to 4,600 new grants this year) by reducing the length and the rate of growth of the average award, and favouring institutions with lower financial overhead, or "indirect costs". Claiming that the "crisis" in health research "has been overstated", the House bill offers the reforms as a way to "bring more stability and predictability to NIH's current fiscal situation". Although Senate staff say their language will be much more "muted", it appears likely that reforms of one sort or another will become law. NIH have formed ten committees to suggest how the institutes should implement the congressional language. Their reports are due by mid-October.

Christopher Anderson

More suitors from abroad

London

IN the latest in a series of long-term investments by overseas drug companies in UK university-based research, Eisai, an expanding Japanese company, last week announced that it will spend £50 million over the next 15 years to set up a new neurosciences research centre at University College London (UCL).

The move has been welcomed by the UK scientific community but at the same time has reawakened criticism of British companies, which show little sign of emulating their foreign competitors' large-scale investment in universities.

The centre will be housed in a new building, to open in 1992, concentrating on basic research into the factors underlying diseases of the central nervous system such as Alzheimer's and Parkinson's. Short-term commercial benefits are unlikely, but any patent rights will be held by Eisai. The centre's 30 staff will be Eisai

employees, and so will need approval before they can publish research data. But Derek Roberts, provost of University College London, does not expect any problems, because Eisai has a good record of allowing employees freedom to publish.

Roberts points out that Eisai chose to site the new centre at UCL, rather than at a less expensive 'green field' site, because it wishes to gain from the exchange of ideas with other academics on the campus. A restrictive policy of commercial confidentiality would soon subvert this aim.

Professor Denis Noble, president of the academic pressure group Save British Science, says the Eisai investment is part of a welcome trend towards closer relationships between university researchers and overseas companies. But he says Eisai's "enlightened" attitude is not shared by British companies, which prefer to use universities strictly for contract research aimed at specific and short-term goals.

Roberts says that UCL finds it "singularly difficult to persuade British companies to put money into bricks and mortar". Little money for new buildings is forthcoming from government, through the Universities Funding Council, so the best option for universities wishing to expand their research facilities is to interest overseas companies. UCL already hosts a pharmacology research centre funded by the Swiss company Sandoz, which opened in 1985. Sandoz leases laboratory space from the college, providing money that has allowed the chemistry department to move to a new building.

Professor David Smith, from the University of Oxford's pharmacology department, another beneficiary of major overseas investment, is also disappointed with the response of British industry. In the mid-1980s, faced with declining government funding, his department began looking for increased industrial sponsorship. But Smith says that of 23 companies that attended a workshop in 1986 to hear about the department's work, only two were British. The Oxford department eventually signed a seven-year £20 million agreement with the US company Squibb (now Bristol-Myers-Squibb) in 1987, to finance a new building for the department and run a number of research projects. Under the Oxford-Squibb arrangement, researchers are not Squibb employees. Squibb files for any patents on the results of research, but the university receives a royalty on any commercial gains.

UK pharmaceutical companies argue that they already contribute to the British universities through the tax system, and point out that companies — both British and foreign — like to be seen to be supporting local research when they are operating in overseas markets. As an example they note that the British company Glaxo last year provided £15 million to set up a neurosciences centre at the National University of Singapore for research into dementia and Parkinson's disease.

For overseas companies, there is also a direct financial advantage that may offset investment in UK universities: drug companies may charge the National Health Service more for their products if they have substantial research activity in the United Kingdom. British companies have no such incentive to invest in university research.

But there does seem to be some genuine dissatisfaction within the British pharmaceutical industry over arrangements for research in UK universities. John Griffin, director of the Association of British Pharmaceutical Industry, last week told the British Pharmaceutical Conference in Cardiff that universities are charging inflated overheads on research contracts to subsidize 'lame duck' departments.

Peter Aldhous

US CANCER EPIDEMIOLOGY

No Sellafield effect in US study?

Washington

A two-year study to be released this week has found no increased incidence in cancer mortality around nuclear facilities in 107 US counties, but researchers warn that the resolution of the data may be too coarse to draw firm conclusions.

The study, conducted by a team at the National Cancer Institute (NCI) led by John Boice, was begun in 1987, after a similar British study published in this journal suggested that there might be a higher incidence of childhood leukaemia near nuclear installations (see *Nature* 329, 499; 1987). Subsequent studies, including one published this year that provided evidence of a cluster of cases of childhood leukaemia in the vicinity of the Sellafield nuclear plant (see *Nature* 343, 679; 1990), have raised more questions than they have answered.

The NCI study seems likely to be no exception. "From the data at hand, there was no convincing evidence of any increased risk of death from any of the cancers we surveyed due to living near nuclear facilities", Boice stated last week. But he also said that the size of the areas from which data were collected may be too large to detect small pockets of cancer immediately around nuclear plants.

In the 1987 British study, David Forman and colleagues from the Imperial Cancer Research Fund Cancer Epidemiology and Clinical Trials Unit, Oxford, used local authority areas (LAAs) as the units of geographic location of the surveyed populations. Forman's team used only LAAs with at least one-third of their population living within 10 miles of a

nuclear facility. Many of the LAAs had two-thirds of their population living within six miles of the installation.

The US counties used in the NCI study are much larger. The NCI team used county mortality records, rather than those located by zip code (which tend to represent much smaller areas), because they were the only complete record to which the researchers had access at the time. "We recognized that it was a problem, but [counties] were the smallest unit for which death data was available", says team member B.J. Stone. "If we had had a choice, we would have gone to census-tracked data." For childhood leukaemia, the NCI study found that mortality risks were slightly higher before the start-up of a nuclear plant in the county than after. For other forms of childhood cancer, the reverse was true. But in both cases, the change in risk is so small (1.08 to 1.03 in the first case, 0.94 to 0.99 in the second) and the counties were so large, that the researchers were not able to attribute it to the presence of the facility. The conclusions of the report recommend a more detailed study.

Although no "patterns jump out at you", says Stone, "there are some plants that are significantly high and some that are significantly low. You do have this feeling that you should go back to certain areas [and do a more detailed analysis] because you feel uneasy." Given the uncertainties in the NCI report and the interest of Senator Edward Kennedy, one of the chief backers of the study, a further project appears likely. Christopher Anderson

Radical computing in Japan

Tokyo

NEURAL, biological and optical computing are among the radical forms of computing to be taken up by a new international institute to be set up in Japan with the backing of the private sector. The institute is the work of the Japan Technology Transfer Association (JTTAS), a non-profit organization affiliated with the Ministry of International Trade and Industry (MITI), and has already won support from a large number of Japanese companies.

The aims of the International Institute of Novel Computing (IINC), as it will be named, appear to overlap with the so-called sixth-generation computer project which MITI is expected to launch in 1992 (see *Nature* 345, 279; 1990). But backers of IINC say the two projects are not directly related.

JTTAS, which is affiliated with MITI's Agency of Industrial Science and Technology (AIST) but funded almost entirely by private industry, began soliciting contributions last April from private companies for a feasibility study for IINC. The association's activities initially angered MITI's machinery and information industries bureau which is promoting the sixth generation project. According to Taizo Nishikawa of the bureau's industrial electronics division, JTTAS was giving the impression that the IINC project is officially backed by MITI when in fact it is not (part of the problem was that JTTAS is affiliated to a different section of MITI).

UK RESEARCH

Call for increased polytechnic spending

London

THE UK Polytechnics and Colleges Funding Council (PCFC) should increase its spending on research from the current £30 million a year to £58 million in 1991-92, according to the report from a PCFC-appointed committee, released today (20 September).

The research committee was set up in April last year by the newly formed PCFC to examine the future role of research in the polytechnic and college sector. Its chairman, Oscar Roith, chief scientist at the UK Department of Trade and Industry, says that many committee members were at first sceptical of the value of polytechnic research. But he claims that all are now convinced that research is "a key component" of polytechnics and colleges.

A survey by an independent team of consultants found that the sector's external research income exceeds that from PCFC by 1.7 to 1. The committee believes that increasing PCFC's spending on research would attract more research funds from industry and the research councils.

But Hideo Aiso of Keio University, the chairman of IINC, says that there is no real conflict with MITI. The two projects, he says, are like "two vectors" going in different directions although it is possible they could merge in the future. (Oddly enough, many of the academic advisers for IINC are also advising MITI on its project).

Aiso says that previous national computing projects, such as the fifth-generation computer project, have tended to set their sights on making a particular piece of hardware, something which is necessary to get backing from the Ministry of Finance. But IINC supporters, such as Shunichi Amari of Tokyo University, one of Japan's leading experts on neural computing, see a need for a more flexible approach that will allow more basic research and may also encourage the participation of foreign researchers. They think the best way to achieve that goal is to avoid government funding at least in the initial phase of the project.

Nine companies, including Sanyo, Hitachi, Nippon Steel Corporation, Mitsubishi Electric and Nissan, have already agreed to contribute ¥1.8 million (\$13,000) a year towards the IINC feasibility study, which will last "about two years", according to Aiso. And many more, including Toyota, NEC, IBM Japan, Honda, Fujitsu, Sharp, Sony, Tokyo Gas Co., Japan Railways and Nippon Telegraph and Telephone Cor-

Polytechnics and colleges received only £6.7 million in research council grants in 1988-89 (about 4 per cent of the councils' annual research grant expenditure).

To increase this, PCFC is to set up a working party to look at the polytechnics' relationship with the research councils. Roith says that polytechnic researchers often do not apply to research councils because they believe university applicants are favoured. But Roith says that the research councils assured his committee that this was not the case.

Nevertheless, universities do have a real financial advantage. Research council grants provide only for the direct costs of research, but polytechnics receive no government funds for research overheads. Universities, by contrast, benefit from the 'dual support' system, where research council grants are supplemented by the research component of the Universities Funding Council (UFC) block grants. In 1988-89, universities spent about £770 million of their UFC income on research.

Peter Aldhous

European body gearing up

London

JOHN Lake, formerly head of science with the UK Agricultural and Food Research Council, is to become the first director of the European Environmental Research Organization (EERO). EERO was conceived as an analogue to the European Molecular Biology Organization, to support basic research in environmental science. It has about 40 members, all eminent European environmental scientists, and a small permanent office in Wageningen in the Netherlands.

Lake says that EERO-supported research must be led by the opportunity of making new scientific discoveries, rather than responding to political demands. Applications for EERO's first round of up to 15 fellowships have just closed. Lake hopes to increase EERO's current annual budget of 2.5 million ECU (1 ECU = £0.70) by "several fold" over the next few years, raising funds from European governments and charitable foundations. The Dutch government and the Volkswagen Foundation have already given support.

Peter Aldhous

poration are shortly expected to join, says Isao Idota, managing director of JTTAS. JTTAS's final target is 100 companies.

Twelve subcommittees each with about 20 academics and industrialists have been established for the feasibility study and they have already begun regular meetings. The subcommittees, headed by leading academics in each field, cover neurocomputing, biocomputing, optical computing and parallel computing as well as several other fields. Thus, the research areas overlap in many respects with the sixth generation project. But Aiso does not expect all of these areas to be covered when the institute finally opens.

Each subcommittee has members from the companies supporting IINC and also several foreign "observers" who will be kept informed of subcommittee deliberations and will be tapped for advice. Among them are Terence Sejnowski of the Salk Institute in the United States and Harold Szu of NASA.

Aiso and Amari say they are determined to make the institute truly international. There have been several recent attempts by MITI to set up so-called "international" institutes but they have failed to draw support from foreign companies and have been unable to recruit significant numbers of foreign researchers.

Aiso and Amari say that the solution may be to establish an institute in the United States and Europe first and only later set one up in Japan.

David Swinbanks

WHO calls the shots

Munich

THE World Health Organization (WHO) and several other international organizations will next week launch an ambitious campaign for universal vaccination of children against diseases such as polio, tetanus and measles at the 'Children's Summit' in New York City. The creators of the 'Children's Vaccine Initiative' say that this will be the first time a broad network of agencies and world leaders will have given their support to the cause of better vaccines, which are the most cost-effective way of saving lives in the developing world.

WHO, the United Nations Development Programme (UNDP) and the United Nations Children's Fund (UNICEF) have joined forces to back an effort that they hope will attract a minimum of \$15 million a year over ten years for vaccine research and development. An expected 70 heads of state, including Soviet leader Mikhail Gorbachev and US President George Bush, will pledge their countries' support at the summit on 29–30 September.

The 'New York Declaration' sets goals of reducing the number of doses of vaccine required for immunization; combining many antigens in a single vaccine; making existing vaccines more heat-stable and affordable; and developing new vaccines against, for example, diarrhoea and acute respiratory infections.

An earlier goal — that of developing a single-dose, preferably oral "children's vaccine" that could immunize children against all endemic diseases — was dropped at a pre-summit meeting of WHO, UNDP and UNICEF experts in New York on 9–10 September.

According to Friedrich Deinhardt of the University of Munich, chairman of the WHO Scientific Advisory Group of Experts (SAGE), it was thought impossible in the near future to use a single vaccine carrier, such as a vaccinia virus, to carry antigens against as many as a dozen diseases. But, says Deinhardt, the use of existing techniques will allow the development of combination single-dose vaccines that would reduce the number of vaccinations necessary to immunize each child, thereby increasing effectiveness and reducing cost.

WHO has succeeded beyond all expectations since it began vaccinating children in 1974 under the Expanded Programme on Immunization. Whereas just 5 per cent of children in the developing world were being vaccinated in 1974, the programme now vaccinates 66–81 per cent of children against deadly diseases such as measles and polio.

Nevertheless, advocates of the Children's Vaccine Initiative are not satis-

fied with the results of the Expanded Programme on Immunization. "Not a single new vaccine has been introduced in the developing world" since the programme began, says Barry Bloom of the Albert Einstein College of Medicine in New York.

And the vaccination programme is still plagued by technical problems, especially that of keeping the vaccines cold from the time they leave the manufacturer to the time they arrive at their destination, often in the tropics.

Even after next week's anticipated rousing start, the initiative will face two large challenges. For one, persuading researchers to work on vaccines will not be easy. "No one gives a prize for making polio vaccine more heat-stable", says Deinhardt. But Bloom, more of an optimist, says that the scientific problems might be interesting enough to catch the attention of researchers. "It's not just pickling a new bug, grinding it up and jabbing it into people", Bloom says. Instead, the research might require "using genetic engineering to make the vaccines less sensitive to heat", he says, "a project which is not trivial." The other challenge

is raising money. Even though \$150 million sounds an impressive amount, Deinhardt says it is little more than "seed money" to get researchers and companies started on vaccine research. Developing a modern vaccine can cost as much as \$20–\$100 million, including expensive animal and human trials. According to Jim Sherry of UNICEF, US industry is ready to play a large part in developing better vaccines, but only if someone else pays the bill.

One likely target for the Children's Vaccine Initiative fund-raisers will be the US government. The list of supporters of the WHO Programme for Vaccine Development, an adjunct to the Expanded Programme on Immunization, does not include the United States but does include Australia, Italy, Japan, Norway, Sweden, Switzerland, the Rockefeller Foundation and UNDP.

US Senator Bill Bradley (Democrat, New Jersey) recently introduced a bill into the Senate to support the Children's Vaccine Initiative with as much as \$125 million a year, although the bill has little chance of becoming law. Nevertheless, Sherry believes that finding support will become easier for researchers who put forward good proposals to US agencies such as the National Institutes of Health.

Steven Dickman

GLOBAL WARMING

France comes cleaner than clean

Paris

FRANCE last week joined the list of countries preparing to take action to reduce greenhouse gas emissions. In a memorandum addressed to the Presidency and the Commission of the European Communities, the French secretary of state for the environment, Brice Lalonde, has declared that France will reduce levels of carbon dioxide emissions to "less than 2 tonnes per capita per year" in the next 25 years. The present emission level of carbon dioxide in France is about 2.3 tonnes per capita per year.

According to a spokesman at the ministry of foreign affairs in Paris, the announcement has been made to help advance negotiations at the ministerial meeting that will follow the Second World Climate Conference to be held in Geneva at the end of October.

Within the environment ministry — which has just seen its annual budget almost double — a spokesman described the French proposal as "ambitious". But France is not a major producer of greenhouse gases, contributing only 1.7 per cent of world carbon dioxide emissions and about 2 per cent of all greenhouse gas emissions. Neighbouring West Germany contributes 3.3 per cent and the United States, 23 per cent of world carbon dioxide emissions (1989 OECD figures).

Energy production in France causes relatively little atmospheric pollution. France has few fossil fuel resources and, following the oil crisis of the 1970s, successive governments backed expansion of the nuclear energy programme. Now only about 9 per cent of the nation's energy needs are met from coal, compared with 32 per cent in Britain and 27 per cent in West Germany. By contrast, some 30 per cent comes from nuclear generators, compared with 7 per cent in Britain and less than 12 per cent in West Germany.

Over the past decade, France's reliance on coal has dropped by 40 per cent, while nuclear energy production has more than tripled.

Now, with the nuclear industry entering a period of decline, the Gulf war could have provoked new investment. But the industry minister, Roger Faroux, has said that he intends only to continue the present programme of reactor construction and has no plans for expansion. Instead, a more or less shelved programme to develop alternative energy sources will be revived, while new measures have been announced to promote energy saving. Of these, extra money will be available to develop a 'clean' car. Transport accounts for almost 40 per cent of the nation's carbon dioxide emissions.

Peter Coles

Stanford argues over costs

Boston

WITH the highest research overhead costs in the United States, Stanford University and its outspoken president, Donald Kennedy, have long been at the centre of the tortuous debate over the standards by which Stanford and other universities calculate overhead charges to the government when they receive federal research grants. Last week, in a new twist to the story, the university revealed that three separate investigations into the university's practices for recovering overhead costs associated with federally-sponsored research are now under way. In addition to Stanford's own "internal audit", branches of Congress and the Defense Department will review the school's practices, with millions of dollars of research funds potentially at stake.

The most explosive investigation is being led by the Office of Naval Research (ONR), the branch of the Defense Department responsible for negotiating the overhead cost rates with Stanford on behalf of all federal agencies. An internal memorandum from a field representative of the ONR that came to light last week accuses Stanford officials of possible "abuse" and "distortion" in their calculation of overhead costs. The letter concludes that "Stanford engaged in fraudulent acts, and, at a minimum, false statements and false claims". Curiously, senior officials at ONR say that they never received the memorandum, which is dated 6 March 1990. Both Stanford and ONR officials claim to have learned of the accusations only recently when the memorandum surfaced as part of a request under the Freedom of Information Act from a local California newspaper. Because of the seriousness of the accusations, ONR has launched a full-scale investigation.

The issue is complex and divisive well beyond the specific confines of the ONR investigation, however. Congressman John Dingell, whose congressional subcommittee has also now begun to investigate the overhead issue, informed Kennedy last month that "escalating overhead rates" at Stanford and other universities have prompted his committee's investigation, which will begin with Stanford but will ultimately review practices at other US universities.

Congressional staff members say that the investigation seeks to determine "exactly what the government is paying for" when it allots hundreds of millions of dollars in overhead charges. Stanford alone, for instance, received some \$91 million in overhead costs last year.

Not surprisingly, university administrators, facing rising energy and operating costs, are conscious that the fate of their institutions is bound up in the overhead

issue. As for specific charges against Stanford, Kennedy stated last week that university staff responsible for indirect cost matters "enjoy excellent reputations in their professions" and will "cooperate fully with all investigations". But he said that university officials will not comment on the allegations until the investigations are complete.

Stanford faculty members protested strongly last spring that Stanford's rising overhead cost rates — now 78 per cent — were affecting their ability to attract federal grants. Competing researchers, they maintained, were "underselling" them because competitors could conduct the same research at other institutions for less total cost to the government.

Under the current system, universities are allowed to recover overhead, or indirect costs, associated with their researchers' grant proposals through a complex set of guidelines. These indirect costs are computed as a percentage of a modified version of the total direct costs associated with a research project; they can include charges for the university's costs of utilities, building maintenance, the depreciation of new facilities, library use and other administrative costs. Thus, if a Stanford researcher's federal grant has a "modified total direct cost" (for researchers' salaries and the like) of \$100,000, the federal agency dispensing the grant will be asked to pay \$178,000 to cover Stanford's share of indirect costs. By comparison, indirect cost rates at MIT, Yale and Columbia are 62 per cent, 68 per cent, and 74 per cent respectively.

As high as those indirect cost rates sound, Larry Horton, Stanford's associate vice president and director of government relations, emphasizes that the percentages themselves are misleading. The rates, he points out, are applied only to a certain portion of a given federal research grant. Thus, Horton maintains that, of total federal funds allotted to Stanford for research, the government actually paid some 43 per cent in overhead charges. However, many researchers still believe that the indirect cost rates are out of control, and government officials complain that many private foundations, such as the American Cancer Society, refuse to pay overhead costs, leaving federal funders to shoulder a disproportionate share.

The controversy surrounding the issue of indirect costs is a longstanding one. Nonetheless, all involved in the current controversy at Stanford predict that the newest round of investigations will once again bring the issue prominently before the public and could provoke new limitations on the indirect costs deemed admissible from universities to the federal government.

Seth Shulman

GE sees patent storm rising

Washington

WHO first thought that isotopically pure diamonds might be the best room-temperature heat-conductors ever created, with a host of possible applications in the electronics industry? General Electric (GE) took credit for the manufacture of the first such pure diamonds at a press conference earlier this year. But Russell Seitz, a Cambridge (Massachusetts) materials scientist, claims that he predicted the high room-temperature thermal conductivity of isotope-enhanced diamonds in a 1975 patent, and told GE researchers about it in 1986.

Most scientists might turn to their lawyer when they suspect someone has borrowed their ideas, but Seitz has instead enlisted the aid of bestselling techno-thriller author Tom Clancy, who used Seitz's predictions about diamonds in *The Cardinal of the Kremlin*, and a host of famous scientists to persuade the GE board of directors to give him some credit for the work.

In a letter to be sent to GE this week (with a covering letter from Clancy), physicists Philip Morrison and Richard Wilson from Massachusetts Institute of Technology and Harvard respectively, join such notable researchers as artificial intelligence pioneer Marvin Minsky and former presidential science adviser George Keyworth in arguing Seitz's case. "In denying Seitz's seminal role in the development of mono-isotopic diamond . . . , GE has not only failed to live up to its own reputation as an ethical firm, but has transgressed the norms of professional scientific conduct", the letter charges.

GE board members have not yet seen the letter but others at GE reject Seitz's claim. Walter Robb, senior vice president for corporate research and development, says that Seitz is one among many who speculated on the effect. "I suppose in the press release and technical article we could have listed all 10 or 20 authors that commented on this effect . . . if we had mentioned a number of them he (Seitz) probably would not have been in the list".

Seitz says that he described the effect discovered by GE at a 1987 talk attended by GE scientists. But GE scientist William Banholzer says that "If he did make such a prediction, it was nothing more than speculation . . . He's had three years to publish it in a refereed journal. Where is it?"

Seitz and GE researchers will have a chance to reconcile their differences next week at a Washington, DC, scientific meeting at which both will present papers. Clancy will be giving an after-dinner speech and Seitz hopes it will be one "congratulating GE" on giving recognition to his contribution.

Christopher Anderson & Alun Anderson

Top names at last

Washington

AFTER more than a year's vacancy in the top post at the US National Institutes of Health (NIH) and a protracted search for a new US National Science Foundation (NSF) director, the administration last week finally revealed its choices.

Bernadine Healy, a nationally known cardiologist, is to head NIH, and Walter Massey, vice-president for research at the University of Chicago and Argonne National Laboratory, will run NSF, assuming that there are no snags in the congressional confirmation process.

Healy has criticized NIH for underrepresenting women in clinical trials, paying too little attention to women's health issues and neglecting women researchers when selecting managers.

Massey, a physicist, is a member of the National Science Board and is vice president of the American Physical Society. He has recently taken a special interest in issues of technology transfer from research to industry. Christopher Anderson

HUBBLE SPACE TELESCOPE

Upside down rod

Washington

AN investigative team assembled by the National Aeronautics and Space Administration (NASA) to find the cause of the Hubble Space Telescope's optical distortions last week fingered the culprit.

A measuring instrument known as a metering rod may have been used upside down, aggravating a separate problem in which reflections from a cap placed at the end of the rod sent false signals that confused the technicians shaping the mirror, the team said.

"Aspects of th[is] scenario have been reproduced with an interferometer and metering bar/end cap simulations, and the end results indicate that the scenario as described is probable", team leader Lew Allen, director of the Jet Propulsion Laboratory, said in a statement.

The metering rod is used to set precisely the distance between elements of an optical device known as a null corrector that was used to help shape Hubble's main mirror. A 1.3 mm error appears to have arisen when technicians fitted an end cap with a pinhole at a position 1.3 mm beyond the end of the solid metering rod, to ensure that the reference beam was reflected off the very centre of the rod's end. However, the end cap was itself shiny and the light beam seems to have been reflected off the cap, rather than travelling through the pinhole.

The fact that the metering bar seems to have been used upside down, with its intended reflective end away from the light beam, only made the situation worse.

Christopher Anderson

Japan gets its act together

Tokyo

THE Japanese government is at last trying to put together into one coordinated whole its various efforts to contribute to international plans to sequence the human genome. Alongside an expansion in funding for genome-related research, the Council of Science and Technology, Japan's principal science policy-making body, will try this week to harmonize the competing activities of various government ministries and agencies.

Japan's financial commitment to research on the human genome has so far been small, and has provoked complaints from James Watson, director of the US National Institutes of Health Center for Genome Research (see *Nature* 342, 463; 1989). Budget increases now under way will still leave Japan with a small contribution in relation to its gross national product, but nevertheless represents a small victory for Japanese scientists.

The Ministry of Education, Science and Culture (MESC) is to boost funding for grants from ¥280 million to ¥500 million (\$3.6 million) per year, following the completion of a two-year preparatory study put together by Kenichi Matsubara of Osaka University. The ministry has also requested ¥850 million (\$6.2 million) over five years to establish a new Genome Analysis Centre at the Institute of Medical Science at Tokyo University. But the centre, which MESC hopes eventually will have a staff of about 20 people, still faces a difficult future because of the freeze on the appointment of new government employees. MESC's plans for the centre are in fact extremely ambitious, as the ¥850 million is only the beginning of a much larger expenditure, and MESC wants to supplement the activities with new posts at universities throughout Japan.

A further problem, according to Matsubara, may be a conflict with the Science and Technology Agency (STA)'s plans to support related work at the Institute of Physical and Chemical Research (RIKEN). Masato Chijiya, director of STA's Life Science Division, says that such an overlap is one of things that will have to be sorted out by the Council of Science and Technology and a committee of ten scientists headed by Wataru Mori, former president of Tokyo University, that will be appointed by the council on 21 September.

STA plans to expand its budget only slightly — by about ¥100 million (\$1 million) — for genome-related research next year. A total of ¥542 million (\$4 million) has been requested for RIKEN, of which ¥303 million will go towards development of automatic DNA sequencing machines and to DNA sequencing.

The remainder goes to a gene bank which is already under development at RIKEN's Life Science Centre in Tsukuba.

Other ministries are also climbing aboard the genome bandwagon. The Ministry of Health and Welfare is requesting ¥400 million, up from ¥200 million this year, for a project to analyse and sequence oncogenes. And the Ministry of Agriculture, Forestry and Fisheries is requesting ¥610 million for fiscal year 1991 to begin sequencing the rice genome. For this project, which is expected to last seven years, ¥155 million is allocated to DNA sequencing and research to be carried out largely at the ministry's National Institute of Agrobiological Resources in Tsukuba, ¥326 million is for mapping that will be farmed out to private companies, ¥100 million is for equipment and facilities, and ¥16 million will go to study of the establishment of yet another DNA bank.

Still to join the fray is the powerful Ministry of International Trade and Industry (MITI). Michio Oishi of Tokyo University is trying to win support from MITI and private industry. Japanese companies do not see much of market in the human genome project itself but are interested in the possibility of developing diagnostic machines for the medical market, Oishi says. It will probably be some time before MITI makes a move but if it does, Oishi is confident that its budget will be larger than that of other ministries.

All of the budget requests are subject to negotiation with the Ministry of Finance but they are unlikely to undergo drastic change. If granted in full, Japan's total government budget for human genome research will still be less than a quarter that of the United States (but well above that of many European countries).

Despite the extra money, Matsubara sees no sign of Japanese funds for the Human Genome Organization (HUGO), which will coordinate the international research effort. The Wellcome Trust in the United Kingdom and the Howard Hughes Foundation in the United States have contributed more than a million dollars to HUGO and one of Watson's main criticisms was directed at Japan's failure to contribute to that organization.

Very restrictive laws on the establishment of foundations in Japan are effectively blocking Japan's ability to contribute to HUGO, according to Matsubara. To establish a foundation, ¥200–¥300 million (\$1.5–\$2.2 million) must first be raised, which actually means raising twice as much because donations initially are not tax free. Then the foundation must operate for several years before it can win tax-free status.

David Swinbanks

GENETIC SCREENING

Law to keep labels off genes

Washington

FEARS that discrimination against those at risk of developing genetic disorders will be a malign result of increasing knowledge about the human genome have prompted a bill introduced last week by Representative John Conyers (Democrat, Michigan).

The Human Genome Privacy Act, the first of its kind anywhere, promises to give individuals the right to privacy on information about their genetic make-up. Discrimination based on an individual's genetic diagnosis and screening records would be prohibited in areas such as employment, insurance and education.

Cases of 'genetic discrimination' have already been documented. According to Paul Billings, vice chairman of the department of medicine at the Pacific Presbyterian Hospital in San Francisco and a prominent supporter of the Privacy Act, individuals have been denied health insurance and employment because they were known from genetic evidence to have disorders such as Charcot-Marie-Tooth muscular atrophy and the neurodegenerative disorder Friedrich's Ataxia.

Asymptomatic carriers of recessively inherited disorders including sickle-cell anaemia and Gaucher disease, a disorder of lipid metabolism, have also been refused employment because of misguided fears that they themselves were susceptible to the disease.

The new act would prevent the disclosure of genetic information by government agencies, contractors or grant-recipients without an individual's written consent. Exceptions would be made in cases of medical emergency and criminal investigation. Conyers, chairman of the government operations committee, has won widespread backing within the scientific and religious communities for the legislation and expects bipartisan support in Congress.

Long-time adversaries Jeremy Rifkin, president of the Foundation on Economic Trends, and W. French Anderson, a leading researcher into gene therapy at the National Institutes of Health (NIH), both find themselves backing the initiative.

Rifkin hopes the act will serve as a model for similar legislation soon to be introduced in about 20 countries, mainly in Europe. Although the proposed US legislation applies only to government agencies and their affiliates, "it is important that government establishes the standard by which private industry will then respond", Rifkin said last week at a press conference held to launch the act.

Anderson was also expected to speak at last week's press conference, but withdrew at the last minute because the Department of Health and Human Services

"denied me permission to go as an NIH scientist", he said. Nevertheless, Anderson affirmed that he "strongly supports the concept behind the bill" and its introduction as a "means of initiating public discussion".

Billings says that "the variability of genetic disorders and the fact that some of them are not particularly severe are not appreciated by the general public or by the people who are making policy decisions for employers, insurers or even adoption agencies". Congressional hearings of the Human Genome Privacy Act will begin next spring.

Kevin Davies & Diane Gershon
SUPERCOMPUTERS

London centre in funding row

London

ONE of Britain's three academic supercomputing centres may be forced to cut back its services, because of a dispute between the Department of Education and Science (DES) and the University of London over the centre's funding. The DES Computer Board has told the University of London that funding for the University of London Computing Centre (ULCC)'s Cray supercomputer must be reduced from almost £2 million to £1 million a year, but the university is reluctant to foot the bill. The board also wants to cut funding for the centre's smaller Amdahl computer by almost £900,000.

Together with a similar-sized centre at the University of Manchester and the larger Atlas facility at the Science and Engineering Research Council's Rutherford Appleton Laboratory (RAL), ULCC provides a national supercomputing service for the UK academic community. But ULCC currently costs DES about twice as much to run as the Manchester centre.

ULCC's actual operating costs differ little from those in Manchester — both run a large supercomputer and a second-string machine. But the Manchester centre is run as a local computing facility for the university. This means that the university can run the national service at 'marginal cost', simply charging DES for the extra staff, software and maintenance needed to supplement the local service. ULCC, by contrast, is separate from the computing centres at each of London's colleges, so the bulk of its running costs fall to DES.

Professor John Forty, chairman of the Computer Board and principal of the University of Stirling, says that ULCC's costs are "abnormally high" and must be brought down "in the national interest". But this means that the University of London must take on more of the centre's

EDUCATION

University of democracy

Paris

ABOUT 40 Chinese students attended last week the 'Summer University of Democracy' in Paris. For one week the students were able to hear talks on different aspects of democratic civilization given by a dozen leading academics, mostly specialists in law and economics, and Jiaqi Yan, a dissident and former adviser to the Chinese Prime Minister.

The 'university' was financed from funds raised by the French Ministry of Culture on the initiative of the Maison Chinoise pour la Démocratie, which was set up in June 1989 as a focus for efforts to establish democracy in China, following the Tianenmen Square massacre.

Peter Coles

running costs or accept cutbacks. Faced with an accumulating financial deficit (predicted to rise more than £40 million by 1992-93), the university is likely to take a tough line, not least because contributing more to ULCC's running costs would set a dangerous precedent for the other national facilities hosted by London.

London's federal structure also makes it difficult to link ULCC with one of the university's other computing centres and run it as a University of London service with the national service provided at marginal cost. The other centres are attached to individual London colleges and are not provided for the university as a whole.

The Computer Board is expected to make a final decision on ULCC's future funding in October. If the University of London refuses to take more of the load, the board will consider switching some work to the other two national supercomputing centres. But Brian Davies, head of computing at RAL, says "the slack couldn't be taken up on the machine we've got". Professor Frank Sumner, director of computing at Manchester, says the Manchester machines would need upgrading to absorb extra work.

The drive to cut costs in British academic supercomputing contrasts with expanding capacity in other countries. Sumner says that British academics were "far better provided for" than their US colleagues six years ago, but investment in new facilities by the National Science Foundation means the United States is now "way ahead".

Expansion is also under way in West Germany, with the German company Siemens installing new machines in Karlsruhe, Aachen and Hanover over the next six months. German academics benefit from the desire of each of the *Länder* to host a state-of-the-art supercomputing centre, with regional government putting up half of the cost of each.

Peter Aldhous

In defence of taxonomy

In *Nature* of 16 August (Vol. 346, 602; 1990), H. T. Clifford, R. W. Rogers and M. E. Dettmann argued that taxonomists might usefully dispense with the existing massive herbarium collections. We have received many letters criticizing this view. Here we publish those received first; the remainder make many of the points below.

SIR—Clifford, Rogers and Dettmann have exaggerated the problems faced by taxonomic institutes and have misunderstood the role of herbaria. They suggest a solution that displays ignorance and a surprising lack of understanding in professional biologists.

The worry is that their solution may appeal to the uninitiated, and could be taken up by busy administrators and politicians seeking quick remedies to immediate ills. As representatives of the systematic botany community in Australia, we would like to stress that systematics is not merely an exercise in stamp collecting or a naming service for other branches of biology.

Briefly, Clifford *et al.* state that herbaria are becoming choked by ever-increasing numbers of specimens, most of which, in their opinion, have so little value that we would be better off without them; they should, the authors say, be pulped. The principle can be applied to all taxonomic collections. With touching optimism, they go on to suggest that funds and staff-time so saved would be diverted to "taxonomic research proper".

No part of Australia's flora is well known *in toto*, but we probably all know of individual species that are so well-represented that some specimens could be pruned without loss. But even if a specialist were to prune, the saving in curatorial load would be negligible. All the institutes we represent already practise some pruning and quality control of incoming material; some reserve sterile material apart until after publication of results, and disposal seems appropriate. But no case at all can be made for ditching the bulk of the collections.

The lack of understanding of the difference between written records and specimens shown by Clifford *et al.* is little short of stupefying. A description makes accessible a selection, a subset, of the total information that a specimen yields. There is no such thing as a complete description; there will be as many descriptions as there are disciplines studying that specimen, and many of them will not overlap. Yesterday we would have had descriptions of gross morphology, anatomy and palynology. Today, we have electron microscopy and biochemistry in many new and revealing facets. Tomorrow, who knows? No specimens, no information.

Today, systematic biology is being rejuvenated by new and more disciplined ways of thinking; the computer provides

powerful new tools and the predictive power of the resulting classifications — the central aim of the systematist — improved. Without specimens, variation cannot be assessed. The amassed collections of ourselves and our forebears now have new potential in the urgent task of discovering, describing, naming and above all understanding the relationships and biology of the riot of life around us while it lasts. It is time to build on the resources of our collections, not to discard them untapped.

Finally, the authors confuse herbaria (the collection of dried plant specimens) with Herbaria (the institutes that care for and use them). The value and usefulness of herbaria are judged by the number of specimens, the geographical areas covered, the groups represented, the state of their curation and the proportion of 'classical' material mentioned in the literature, including types. But Herbaria are indeed judged in part by the quality of their research, in part by their attitude and accessibility to visiting researchers. The quality of the research is a much more complex mix of factors than Clifford *et al.* allow — published floras, monographs and papers in the scientific literature are the most obvious, but accuracy in the identification of collections derives in large part from long familiarity with the collections. In turn, these identifications are the key to the literature and are thus of crucial importance to all those other disciplines that rely on taxonomists' insight and experience.

It is to be hoped that those concerned with support and management of our biological collections are not misled by the simplistic, short-sighted and ill-conceived ideas put forward by Clifford *et al.* Comprehensive, well-curated collections are essential for the production of high-quality systematic research sought by these authors.

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SIR—Clifford *et al.* raise some interesting points concerning housing collections in the natural sciences. But I will not be jettisoning any part of our herbarium.

First, plants, unlike chemical compounds, are much more complex subjects

where any current description interprets only x out of n characteristics. Experience shows that the next investigator will want to see all the available material, not just an historical account.

Second, we keep well-documented specimens, as well as type(s), because the originally described material may not adequately show the range of variation in species (super-orders are too coarse a taxonomic category) and may not reflect the changing distribution of species.

Finally, reference material is needed to identify enquiries and for educational purposes, and specimens are both historically interesting and aesthetically pleasing.

At a time when many institutions in the public sector are under pressure to provide short-term solutions to cash problems, it is tempting to cast doubt on long-term scientific objectives and commitments to material culture. But with most of the world's species still to be documented, this is no time to weaken our resolve. Rather, natural scientists should focus their skills and what resources they have left in overcoming the mid-term blues. They could start with a look at collecting policies and regional needs.

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SIR—The taxonomic Brave New World outlined by Clifford *et al.* is strange indeed: natural history collections, they say, are not needed because chemists do not store the compounds they synthesize; taxonomy should be based on descriptions and type specimens and should have a rational economic basis.

But one cannot synthesize individuals as a chemist synthesizes compounds. A chemical compound and a herbarium sheet are not comparable entities, as philosophers of science have long acknowledged. To say they differ in degree, not nature, simply will not do.

Descriptions alone, even when accompanied by type specimens, are no basis for the comparative biology of the future. For one thing, descriptions are often poor representations of what is described — and, in this area, taxonomists need to progress. And taxonomists make mistakes.

By way of illustration, species limits now need extensive change in a phylogenetically critical group related to the mangosteen (*Garcinia mangostana*), found from the Philippines to New Caledonia. I know this only because I have access to collections assembled at great cost over 250 years; the descriptions of the species, even recent descriptions, are of little help, but the specimens, and some new characters, are.

Knowledge of plants in the field is important, but well-maintained herbarium collections contain a mine of information. Future monographers are

likely to rely more, rather than less, on herbarium collections for an understanding of some aspects of variation patterns. These collections will more and more represent populations that have become extinct because of man's activities.

It is ironic that a photograph of Kew Herbarium should accompany the article by Clifford *et al.* Almost 100 years ago, a director of Kew, W. T. Thistleton-Dyer, suggested a similar course of action to the one they propose. He accepted variation, but thought only a single specimen of each species, representing the typical morphology of the species, was needed in the herbarium. A number of 'duplicates' were removed from the collection at Kew, and some sent to Berlin — where they were described as new species (B. Verdcourt, personal communication).

Natural history collections are a celebration of diversity, right down to the level below the species, but Clifford *et al.* rightly ask whether we need all the collections. Problems attendant on the storage of voucher specimens are real. Clifford *et al.* might also have noted that many duplicates of one genotype may be taken from a single tree, and that 20 duplicates housed in as many herbaria might be excessive, useful though they may be in evaluating variability.

One wonders if the general solution proposed by Clifford *et al.* is a justification for cutting down on herbarium activities in a particular university department; a rationalization after an all-too-common academic tragedy. Being associated both with a large herbarium and a university department, I believe that rather different kinds of research may be appropriate for the two. But the proposals made by Clifford *et al.* will help neither academic institutions nor the herbaria; taxonomy must first have a rational *scientific* basis, and to destroy the collections will not provide this.

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SIR—I was surprised that three biologists, no doubt experts in their own fields, should appear to have such naive views about the value and methodology of basic taxonomy as to suggest that our collections should be turned over to the garbage collectors. Even a superficial consideration reveals that their suggestion is completely counter to the rigorous methods entailed in scientific research, which should ensure that results are based on repeatable analyses of natural phenomena.

Clifford *et al.* say "Biological specimens may be different from [chemical specimens], but they differ only in degree not in kind". A chemical element or compound can be rigidly defined and shows variation only within certain repeatable limits,

whereas a biological species is of necessity defined according to one type specimen of a species, a species being composed of many individuals showing a range of variation. It is impossible to express the nature and extent of this variation in its entirety and it is therefore necessary to sample it and to keep material for research. Chemicals do not reproduce, neither do they pass on heritable characteristics to their offspring. The destruction of herbarium material, as suggested in the Commentary, would ensure that no one could then verify or refute results which did not appear to agree with the main body of data about, say, a species.

One of the more grotesque suggestions of Clifford *et al.* is that decisions on taxonomic changes should be based solely on published descriptions. Perhaps the best way to show the fallacy of their argument is to consider one particular case.

Epling prepared a revision of the genus *Eriope* from South America in 1936. He was a careful and productive worker, but in this case his research had many defects. He recognized 21 species. If all the material had subsequently been destroyed, I would not have been able to revise the genus, finding that one species had been misinterpreted and belonged to another genus and another had been misinterpreted and belonged to a different family. Of the remaining nineteen, one was better placed in a separate genus, due to characters which Epling had failed to observe, and a further six I was able to reject as one species, as the result of careful analysis of the range in variation of the other material of these taxa available in the form of herbarium data, which Clifford *et al.* would have pulped. I also described a further three new species using material sent to me by other herbaria.

My publication on this topic, which included distribution maps to show biogeographical patterns, would have been impossible without all the herbarium material at my disposal, including not only new but also re-identified materials wrongly assigned by Epling.

As a result of my studies on *Eriope* and related genera, I was able to regroup the taxa into genera that more closely reflected their natural relationships. Nevertheless, my fieldwork has also shown me that I am not infallible, but that some opinions I held at the time of my 1976 revision were erroneous. Thank goodness the herbarium material is there to prove it.

To prepare a data-matrix for cladistic or numerical analysis, it is always necessary to re-investigate characters that may have been ignored or misinterpreted by earlier workers. This would be impossible in the taxonomic wasteland proposed by Clifford *et al.*, or perhaps these authors do not believe in such methods.

Finally, because there is at present a

shortage of funding in the sciences, it is fair and proper to look at which areas need greatest priority of support. But, under these circumstances, I am suspicious of those who, through ignorance, are prepared to back the destruction of a branch of science other than their own. There is, of course, frustration that the classification and description of the approximately 200,000 species of flowering plant is still far from complete. Today, the need for this is ever more urgent, as pressure on natural environments becomes more acute. The fault, however, is not due to the lack of efficiency nor lack of desire to use modern methods, but is due to lack of resources. Science and industry rely on taxonomists, but there is less willingness to acknowledge that the quality of our expertise is inevitably related to resources put at its disposal.

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SIR—There is a gross mischief about that taxonomic research is unrelated to the curation, maintenance and expansion of herbarium collections. It has even been suggested by Clifford *et al.* that it is not necessary to maintain large collections of plant specimens in herbaria, but instead that most collections can be pulped, replacing them with computerized label data. Such a radical proposal may seem sensible and plausible to many of *Nature's* readers, but it is based on unsound logic and a less than full appreciation of taxonomy as a science. Researchers from other fields should know why we are so upset and should appreciate how the proposals put forward by Clifford *et al.* could eventually affect them.

We have all spent time in European herbaria either because our countries did not have adequately named herbarium material, or because our herbaria lacked types and literature. It was only in these large centres that we could complete our work or find solutions to taxonomic problems raised by our colleagues.

Clifford *et al.* suggest that there is no need to collect representative specimens of plants from different localities, at different times of the year, at different stages of the life cycle, of different ecotypes, with different chemical constitution, and so on, and say that it is wasteful to keep voucher specimens for biological and chemical research. Their suggestions would lead to the destruction of the only validating evidence that the specimen(s) used in experiments was correctly identified. For instance, one of us (C.H.S.) recently collected the few surviving fragments of seeds of sophoroid legumes used by chemists to isolate new chemical compounds, only to find that some of the species were not Sophoreae and did not even belong in the family Leguminosae. There were no

herbarium vouchers kept. It is probable that many anomalies in the phytochemical and cytological literature are attributable to misidentifications like these.

The preserved specimen is the only object to which data and information, current and future, applies. Preserving only the information on a specimen label would preserve only the presumed identity of the taxon. Clifford *et al.* maintain that living material can be substituted for dried collections. This is nonsense: first, it may not be possible to re-collect the species in the field, and second, comprehensive dried collections from the total distribution range give a far better idea of a species' variability than limited live collections. Further, large, living plant collections would take up expensive greenhouse space and garden facilities. So what money would have been saved?

What stability there is in the classification system is there because the material is available for restudy, fresh material is added from remaining populations, and newer techniques are applied to gather more data. This enhances the scientific value of the material that has been studied as it bears the determinants (identification labels) of generations of revisers and links it to their and other scientists' publications.

The science of plant biogeography, which depends on the accurate identification of plants and a knowledge of where they were collected, would cease to be a viable discipline if herbarium holdings were destroyed. The demise of biogeography would make it difficult to reconstruct past climates given that many plants are sensitive trackers of climatic change. An important tool for the study of global warming would no longer be available. A similar case could be made for lichens, which are valuable in monitoring atmospheric pollution, and algae, which can monitor water pollution.

The literature-based taxonomic system proposed by Clifford *et al.*, which decides, for example, nomenclature priority on published descriptions and not type specimens, is unworkable. A specimen allows renewed interpretation and description; a published description is only one person's, sometimes prejudiced, interpretation.

The importance of making both type specimens and associated herbarium material available for study is shown by the example of the legume species *Astragalus setiferus*. If only the type specimen had been preserved we would probably not know, as we do now, that this species actually belongs to *Cornulaca* in the Chenopodiaceae. It was the critical study of more complete later collections that enabled the species to be correctly placed. There is also the problem of taxonomic uncertainty, best illustrated by the publication in the 1890s of four different classifications of the tribe Asclepiadeae

(Asclepiadaceae). Any reassessment of these differing classifications will have to be based on a study of the same material.

The preservation of well-cured plant specimens in dried or liquid state in herbaria is an essential and basic scientific goal. If there is "massive duplication" that exists for some taxa in some large herbaria then there seems to be a good case to distribute that valuable material more equitably around the globe. Although some large European and US herbaria may be "creaking at the seams", many countries in the Southern Hemisphere do not have a national collection of preserved plants. In such countries, naming is often done by matching against the limited collections in universities and government departments. Despite this, the naming service is probably one of the most important taxonomic services provided to farmers, doctors, hospitals, herbicide companies, gardeners, foresters, environmentalists, conservationists, entomologists, mammalogists, ethnobotanists and ecologists in developing countries.

The real issue at stake is not that herbaria are overcrowded, or that scientific fashion seems to render herbarium taxonomy redundant or that it will be made superfluous by molecular systematics. Rather, it is that although politicians scarcely question the national and regional preservation of cultural artefacts, they fail to understand the real economic significance of the natural 'artwork' of the world, often the resources for manufactured resources. Herbaria are arks of biological diversity, which should be esteemed for their immense aesthetic, intellectual and scientific contribution to the broader communities they serve, rather than be pulled down and their contents and expertise thrown to the winds.

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SIR—The Commentary by Clifford *et al.* is irresponsible and thoughtless. The authors say that accurate identification of taxa is of prime importance to most biological research, but they devalue the importance of herbarium specimens (except type specimens) as the basis for all botanical classification. Herbaria contain evidence of where a plant occurred and how its appearance varied over time and space, providing physical and biological parameters of the history, biology and relationships of the taxon. It is impossible

to obtain these data other than by examining herbarium specimens. As the plants of the world disappear, herbaria will become a primary means of studying and understanding the principles that govern the fate of those that are left.

Type specimens of species names are essential to fix their position in the nomenclatural system. But they are not necessarily typical of the morphological variation of a species. To be an effective tool for research, a herbarium must contain a geographical and morphological range of each species.

As new methodologies revolutionize taxonomy, herbarium material is re-examined to produce up-to-date classifications. The suggestions by Clifford *et al.* that plants can be re-collected ignores the fact that in many cases the original plant populations no longer exist or have been significantly changed. Moreover, the preserved specimens obviate the need to re-collect rarities, and re-collection is often costly or impracticable. With modern technologies, herbarium seeds have been used to resurrect species extinct in the wild. Many published descriptions are inadequate: a specimen is worth a thousand words. A recent study of the Australian Orchidaceae, for example, revealed that many of the names of the Australian orchids were misapplied by people who had relied on descriptions rather than referring to the original specimens.

We regard our herbaria as the most valuable asset in our broad-based research programmes. In studies of diversity, cytology, plant chemistry, agriculture, forestry and medicine, herbaria are the most cost-effective method of encompassing variation. Improvements in our knowledge depend to a large extent on the quality and selection of materials collected since the previous revision; this applies particularly to unexplored regions. Herbaria, therefore, must grow. The cost of maintaining specimens as part of a broader research programme is relatively small.

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■ Further correspondence on this topic will be considered only if it makes any new points.

Sandpiles as a paradigm of noise

A theory advanced in 1987 as an explanation of the ubiquitous character of noise power-spectra has been partly confirmed by sophisticated measurements of a pile of sand.

Not often, these days, can a theorist expect an analysis to be quickly tested by experiment. In fields such as space research, people are lucky if telling observations materialize within their lifetimes. But the theory of Per Bak, Chao Tang and Kurt Wiesenfeld of the Brookhaven National Laboratory of what they call "self-organised criticality" has been successfully put to the test just over three years after publication (in *Physical Review Letters* **59**, 381; 1987).

Part of the explanation is that the challenge with which Bak and his associates faced experimentalists is at once intriguing and easily comprehended: experimentalists have enthusiastically boiled it down to the question of how the instabilities of a sandpile can be quantified. Bak *et al.* have seen their theory first denied by observation (at the beginning of last year), but now more convincingly supported. The rest of us can expect endless refinements of the sandpile measurements.

Why are they important? Bak *et al.* set out to explain the prevalence of "1/f noise", or the observation that the power spectrum of the noise of a variety of dynamical systems (essentially the Fourier transform of their fluctuations as a function of time) is strictly and inversely proportional to some power of the frequency (f) over a vast range of frequency. The noise of an electrical resistor is well-known to have this property. So does the flow of water in the Nile and the luminosity of a star.

Physically, 1/f noise is consistent with the expectation that smaller fluctuations will be the more common, but there are many other ways in which this might be accomplished, by an exponential decrease for example. There are important connections between 1/f noise and the scale-invariance of phenomena, as in fractal structures.

Why should 1/f behaviour be so common? Bak *et al.*'s explanation is that there is a tendency for large-scale systems to become organized into a state in which they are just on the edge of instability. The energy output from a star, for example, is determined not by the rate of energy production in its core but by that at which energy escapes by convection through the relatively cool layers just beneath the surface. So a small perturbation of the system will bring about a major change in the convection pattern. Fault-systems along which earthquakes recur are in a similar condition.

Whence sandpiles. Sand poured carefully onto a level surface will form a conical pile whose angle is determined by the properties of the sand-grains and the gravitational acceleration. But such a cone is on the edge of instability. Add only a few grains by way of perturbation, and there will be an avalanche — most probably a small avalanche, but, less frequently, a large one.

Three years ago, Bak *et al.* carried out a rudimentary numerical simulation to demonstrate the behaviour of a sandpile. The problem is worked on a square lattice, say in two dimensions. The starting-point is a random pile, which is allowed to reach stability by means of the simple rule that, if the height anywhere exceeds some arbitrary critical value, it is reduced by four units (sand-grains) while each of the four neighbouring sites is increased by one, which may mean that one or more of those sites exceeds criticality. So the process is continued until stability is attained.

Then it is possible to measure the simulated instability by notionally adding a sand-grain at random and measuring the extent of the resulting reorganization by the continued application of the rule, which is nothing but a rule specifying the working of a cellular automaton. Bak *et al.* found that the most common reorganizations were small, but the likelihood distribution of the extents of reorganization in their simulated sandpile was inversely proportional to extent over a range spanning two decades in size.

More sophisticated simulations have since been carried through; more realistic simulations of a sandpile, for example, result from rules specifying their relaxation that depend on the differences between the parameter corresponding to height at one point and at each of its neighbours. But Bak *et al.* seem also to have stimulated experimentalists to hunt for other measurable systems illustrating the principle of self-organized criticality.

For example, Babcock and Westervelt of Harvard University described earlier this year the behaviour of the cell-shaped magnetization domains in a two-dimensional film of iron-garnet doped with bismuth (*Phys. Rev. Lett.* **64**, 2168; 1990). Apparently, a magnetic field perpendicular to the film will yield a stable pattern of cells in which the magnetization is either parallel or anti-parallel to the field, but which may be dramatically disturbed by small increases of the field. Some cells grow at the expense of their

neighbours, with the result that the original stable pattern is much simplified. If the size of the avalanche is the number of cells eliminated (from the arbitrary field of view of a microscope), the likelihood turns out to be inversely proportional to the size^{2,5} or to the time^{2,3} taken for the adjustment to be complete.

But magnetic domains are a long way from sandpiles, which may have an atavistic appeal to experimentalists. Thus Jaeger, Liu and Nagel last year announced (*Phys. Rev. Lett.* **62**, 40; 1989) that, "[a]s children we have played with sand and it is only natural to believe that we can intuit (*sic*) the processes underlying the shape and dynamics of these structures". (In passing, it may be asked why people ready to make such an interesting observation on their motives should also, with such studied inelegance, make a noun into a verb — and why their editors should let them.) The upshot of their observations, with glass beads and rough aluminium oxide particles in rotating drums using parallel-plate condensers to measure the sizes of avalanches, was that the predictions of Bak *et al.* were not verified. Rather, the authors concluded, avalanches are determined by the difference between static and (lesser) sliding friction.

Now, G.A. Held and five colleagues at the IBM research laboratory at Yorktown Heights provide evidence in the other direction. Their trick is to build conical piles of sand (again aluminium oxide particles, but sieved) on the circular pan of an electronic balance and then to add grains singly, using the balance both as a means of measuring the sizes of avalanches and, through a PC, to prevent the addition of further grains while an avalanche is still in progress. (An avalanche carries material off the pan, which is not then weighed.)

The outcome guarantees endless experiments of this kind. For smaller sandpiles, expectations are confirmed; indeed, using the interval between the addition of successive grains as the unit of time, Held *et al.* conclude that the power spectrum (the square modulus of the mass fluctuations) is inversely proportional to the f^2 for sufficiently small (high-frequency) avalanches. But, for larger piles, oscillation between the states determined by static and sliding friction matters. So now, for starters, the hunt is on to define the transition from one regime to another.

John Maddox

Righting the wrong protein

Paul M. Quinton

IN the summer of 1989 the gene for the fatal hereditary disease, cystic fibrosis (CF), was identified and sequenced¹⁻³. Now, barely a year later, two independent groups simultaneously report correction of CF cells transfected with the normal gene. One group (Drumm *et al.*) reports its gene complementation results in *Cell*⁴. The other presents its work on expressing and synthesizing the gene product⁵ and its complementation work⁶ in next week's *Nature*.

Gregory *et al.*⁵ describe *in vitro* and *in vivo* synthesis of the gene product, cystic fibrosis transmembrane conductance regulator (CFTR). Like Drumm *et al.*, they surmised that previous failures to express the gene were due to toxic over-expression caused by a cryptic bacterial promoter in exon 6B of the CFTR gene. Gregory *et al.* solved the problem by incorporating the full length of an open reading frame complementary DNA of CFTR into a low-copy-number plasmid, whereas Drumm *et al.* introduced three mutations into the CFTR gene to neutralize the promoter. Gregory *et al.* went on to synthesize the CFTR gene product *in vitro* in cell-free reticulocyte lysates and *in vivo* with recombinant HeLa cells. To confirm gene expression they developed antibodies to fusion proteins containing peptides encoded by exon 10 and exon 13 of the gene, which labelled protein products of relative molecular mass about 130,000 and 140,000 (*M*_r 130K and 140K) from the *in vitro* and *in vivo* syntheses, respectively. The different molecular masses are accounted for by *in vivo* glycosylation. Although these results give convincing evidence of expression of the CFTR protein, the discrepancy between the apparent small size of these products compared to that predicted for the CFTR protein (which consists of 1,480 amino acids and has a putative *M*_r of 170K) requires clarification.

Transfection

Rich *et al.*⁶ used two independent assays to demonstrate that the defect in cyclic AMP-mediated stimulus-response coupling of chloride permeability in a CF respiratory cell line was corrected in most cells when these cells were transfected with a hybrid vaccinia virus-CFTR cDNA plasmid expression system. Failures were attributed to inefficiency in transfection. Few measurements were possible because of tedious technical demands and the short life of cells after viral infection, but the results from both assays taken together offer convincing evidence that the defect was effectively complemented. Drumm *et al.* corrected the same defect

in a pancreatic tumour cell from a CF patient, using a recombinant retrovirus that produced stable, viable cells and permitted a larger number of measurements using similar assays. Even though these results do not establish how CFTR affects chloride permeability, they do, of course, prove to physiologists what geneticists already knew — that the gene that was identified through 'reverse genetics' is the CF gene.

There are now at least two major routes of research to be pursued directly. First, CFTR antibodies in cells known to express CF defects must be localized cytologically. The many cell types of relatively undesignated function that comprise airway epithelia, and the relative inaccessibility of other equally complicated CF target tissues, may create confusion with respect to antibody specificity in localization studies, but the simplicity of the sweat gland could provide a standard in such assays. The sweat gland reabsorptive duct is a syncytium of cells expressing the clearest and most consistent defect in CF — impermeability to chloride ions — and the sweat gland secretory coil, which characteristically fails to respond to β -adrenergic stimulation in CF, is composed of only two very distinct secretory cells. It seems reasonable to insist that any antibody to CFTR should show binding in these two components of the gland. Antibodies meeting this criterion could then be used to identify target cells in the more complicated tissues of the airways, pancreas and other affected organs. Distinct antibodies meeting this criterion (preferably antibodies to different epitopes of CFTR) will be important in determining if other cells not currently recognized to be affected in CF also express CFTR. Antibody-localization studies seem almost certain to provide insights into the all-important question of the function of the affected protein, which is also at the heart of the second pressing route of pursuit — the biochemical properties and function of CFTR.

On the basis of homology data and modelling, Hyde *et al.*⁷ have argued that CFTR is probably not an ion channel, but rather an ATPase transport protein. The course of further research, and indeed our understanding of the nature of the disease, depends on the assessment of this hypothesis. It predicts that aberrant ion-channel phenomena are secondary consequences of deranged cell metabolism and implies that the disease could be even more amenable to therapy than anticipated if the species transported by CFTR could be identified and controlled. Alternatively, if CFTR is an ion channel or a

closely associated channel regulator, correction will probably require supplementing or bypassing with other components.

Now that quantities of CFTR can be produced, several crucial questions can be answered to define its properties and potential function and thereby distinguish between these two main hypotheses. Initially, we must know if the region containing the most common CF defect (a deleted phenylalanine in the 508 position of the CFTR protein) is a nucleotide-binding fold; that is, does it bind ATP or other nucleotides? If so, is it a catalytic site for ATP hydrolysis? And if it is, is it a transport ATPase or a kinase? If it is a kinase, what does it phosphorylate? And certainly, if it is an active transport protein, what does it transport?

Channel regulation

Even if CFTR does not hydrolyse ATP, nucleotides might still be involved in channel regulation. For example, there is much evidence⁸ showing that the opening of a potassium-ion channel found in pancreatic β -islet cells, heart and skeletal muscle cells, and some neurons, is highly sensitive to ATP or, more probably, to the ratio of cytoplasmic levels of ATP to ADP (energy charge). Airway epithelia⁹ and sweat ducts¹⁰ are characterized by chloride conductances (permeabilities) which decrease markedly with energy deprivation. These observations could suggest that the putative nucleotide-binding fold of CFTR determines the sensitivity of an ion channel, directly or indirectly, to cell nucleotide levels, possibly in a way that protects the cell from electrolyte overloads when it is under stress or short of energy.

Whatever the actual function of CFTR, the two groups that have demonstrated expression and complementation of the CF gene have taken us to another milestone on the route from the genetic defect to effective therapy. Only a few years ago the idea of introducing normal genes into the CF lung to correct its fatal susceptibility to infection was science fiction. Now the accomplishments of these investigators seem to press the fiction inspiringly close to reality. □

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Molecular-scale lubrication

John H. Cushman

It has been known for a few years that layers of fluid just a few molecules thick can acquire solid-like order if sandwiched between stationary, structured surfaces. According to new experiments by Israelachvili and colleagues reported in the *Journal of Chemical Physics*¹, such solid-like order may also exist when the two surfaces are in relative motion. This discovery could be said to mark the birth of molecular tribology, the study of friction and lubrication at the atomic scale.

If two surfaces are brought together to within molecular dimensions in a fluid bath, the interlayer fluid alters its dynamic and static properties. The fluid's phase diagram is changed, its thermal transport coefficients change, it becomes inhomogeneous and may become anisotropic. The properties of the fluid depend in a complex way on the initial structure of the liquid, the structure and commensurability of the surfaces, the surface-liquid potential, the separation of the surfaces, the direction of shear, the rate of shear and the history of shear. The complexity of the fluid film is born out both experimentally and computationally. All this suggests that current theories of lubrication and friction are inadequate. Just as importantly, it suggests that no conceptually correct definition or understanding of a liquid exists when a fluid is confined between two molecularly close surfaces.

In the past few years it has become routine to measure the forces perpendicular to two parallel solid surfaces separated by a molecularly thin fluid film. For film thicknesses (h) less than approximately 10 molecular diameters, the normal component of the surface force (normal stress) oscillates as a function of h with a period of approximately one molecular diameter.

The oscillatory character suggests that the fluid near the surfaces arranges itself in layers parallel with the surfaces and that entire layers of fluid are successively forced from the space between the surfaces as h decreases; indeed, statistical mechanics calculations confirm this notion. It was this layered structure that aroused the curiosity of tribologists. This curiosity led Israelachvili and co-workers to develop instrumentation for measuring the shear stresses a molecularly thin film exerts on parallel surfaces as they slide by one another².

Already the investigators have made significant progress in elucidating the strain-rate/stress behaviour of several simple fluids and mixtures of simple fluids¹. Consider for example the experiment illustrated in Fig. 1. Two mica surfaces bathed in a fluid are forced together under a constant load that is sufficiently large to press all the fluid out of the interlayer region except for a few monolayers. One mica surface is attached to a movable stage by springs and the other surface is held fixed. The stage is set into motion parallel to the surfaces and the shear stress exerted by the fluid on the mica surfaces is recorded. The starting time which is recorded as $t = 0$ indicates when the translation stage begins moving with velocity v , but owing to the yield stress $t = 0$ does not necessarily coincide with the time the mica itself begins to move.

As indicated by the dashed line, if the interlayer separation is sufficiently large (greater than about 10 molecular diameters) the fluid behaves as if it were in the bulk phase — as shear begins the stress in the fluid builds up to a constant value and when the shearing is stopped the stress relaxes at an exponential rate to zero. As illustrated by the curve immediately above the dashed line, all of the fluids studied¹ exhibited liquid or liquid-like behaviour for film thicknesses between 5 and 10 molecular diameters. A liquid-like film is generally associated with non-spherical molecules. The main difference between liquid-like and liquid films is that in the former the stress does not relax to zero when the velocity is set to zero. This is most probably a result of the change in orientation or entanglement of the molecules.

Films of thickness less than 5 molecular diameters take

on a solid-like character, as illustrated by the uppermost curve in Fig. 1. As the stage begins translating, the stress builds linearly while the surface remains in a fixed position. Eventually the yield point is reached, at which time the stress is instantly reduced. The process then repeats. The solid-like character of the film exists only up to a critical velocity, indicated by v_c in Fig. 1, at which point the film becomes fluidic. An increase in

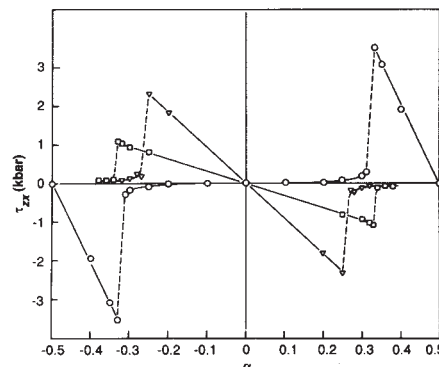


FIG. 2 Shear stress τ_{xz} as a function of registry α for surfaces separated by $h = 2.2\sigma$ (○), 3.10σ (▽), and 4.9σ (□) where σ is the diameter of the fluid (argon) atom. (From ref. 3.)

the velocity of the stage is also commonly accompanied by a decrease in the number of fluid layers existing in the film; this is indicated by the transition in the uppermost curve in Fig. 1. As illustrated by the inset, with certain liquids the sliding starts as liquid-like and becomes progressively more solid-like with increasing elapsed time; this is generally coincident with a decrease in film thickness.

It is interesting to note that the new experimental observations for spherical molecules are qualitatively predictable and explainable from equilibrium statistical-mechanical studies of fluids in micropores^{3,4} and to some degree from early continuum theories of deformation. Consider the plot (Fig. 2) of the stress exerted by an argon film sandwiched between two (100)-planes of a face-centred-cubic crystal, versus the registration of one surface with respect to the other³. The registration is measured by α : when $\alpha = 0$ the atoms on one surface perfectly overlie the atoms on the other; and when $\alpha = 0.5$ they are in one direction fully out of registry.

For film thicknesses less than 5 atomic diameters, the film freezes or liquefies in accordance with the registration of the two surfaces. The linear portion of the 'solid-like' curve in Fig. 1 corresponds to the linear portion of the curves in Fig. 2, as do the yield points. And the saw-toothed portions of that curve correspond to the solid/liquid/solid transitions in Fig. 2.

So the initial state of the fluid is solid and it behaves elastically (that is, its strain increases linearly with stress) until it reaches the yield point, at which time it

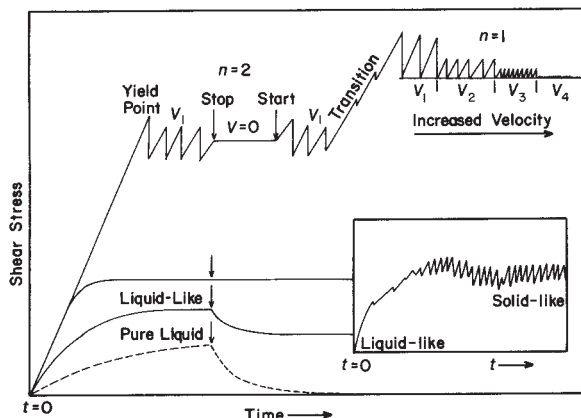


FIG. 1 Shear forces associated with different types of sliding modes as a function of elapsed time for constant load: v is the velocity of the translational stage and n is the number of fluid layers in the film. (Modified from ref. 1.)

liquefies. The surfaces then move past one another until their registration again allows the film to solidify epitaxially. The subsequent yield stresses are slightly less than the original one because the full solid structure of the original is not fully resumed: the time constant for relaxation to the solid state is too slow.

An interesting feature of Israelachvili and colleagues' solid-like curve is the transition which occurred from a film two molecules thick to one a single molecule thick, a change explicable in terms of the increased stability a film gains as it becomes thinner. This change in stability is also evident in the increased slopes and the increased values of the maxima and minima in Fig. 2. The transition is also related to the film's ability to resume a solid-like structure. If the surfaces were held at constant separation, the solvation force normal to the surface would decrease when the surfaces were out of registration. But because it is the load that is held constant in the new work, the surfaces collapse, forcing a layer of fluid out. The solid-state is then more stable and can form again.

The advent of this new experimental tool will go a long way towards developing quantitative data on complex fluid-solid interactions. In this regard, Israelachvili and colleagues have already obtained interesting data on the role humidity plays in lubrication of hydrophilic surfaces by organic liquids. Even small amounts of water (less than 0.01 per cent by weight) can drastically lower friction. The solubility of water in hydrocarbons is very low (50–100 parts per million) and consequently most of the water in a hydrocarbon-water mixture is adsorbed to the hydrophilic mica surfaces creating a slip plane between the water and hydrocarbon. It is this slip plane that is most probably the cause for the drastic reduction in friction upon addition of water to a hydrocarbon. This information may turn out to be extremely valuable in techniques for recovering petroleum from porous media.

Although the new work gives only a glimpse into the complex behaviour of confined thin films, it provides an important starting point for many key experiments in tribology, colloid science, engineering and soil science, as well as physics and chemistry. Developments are awaited with interest. □

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A new thrust at accretion

*Leg 131 shipboard scientific party**

SEDIMENT on the sea floor at a subduction zone, where an oceanic plate is driven below neighbouring continental plate, deforms and may become detached from the underlying basement rock rather than sinking into the Earth's mantle (Fig. 1). Drilling at the Nankai Trough off the coast of Japan (Fig. 2), we penetrated right through the accretionary prism of sediment made by such processes, recovering cores from the overlying accreted sediment and material from the detachment zone and below. The complex results of the tectonic forces at this active continental margin soon became apparent.

The Nankai Trough is the site of subduction of 15-million-year-old oceanic crust of the Shikoku Basin beneath the Japanese arc. Our preliminary seismic survey² clearly showed the frontal thrust fault within the accretionary prism and a deeper seismic reflector representing the décollement surface. The décollement forms the boundary between the scraped-off sediments, which become incorporated into the accretionary prism, and the oceanic plate below, which is subducted back into the Earth's mantle.

From site 808 we obtained a continuous section through 1,290 m of sediments from the middle Miocene (15 million years ago) to the Holocene (the present) at the toe of the accretionary prism. The upper 560 m

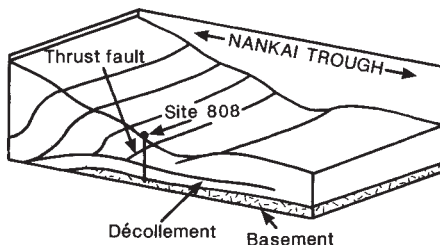


FIG. 1 Schematic of accretionary wedge at an active continental margin.

of sediments are predominantly turbidites, deposited along the trough by strong currents from the Izu-Bonin Arc – Japanese Arc collision zone to the east³ over the past 500,000 years. The lower sediments are more characteristic of open oceans, having accumulated away from the trench in the Shikoku Basin.

The major thrust fault 365 m below the sea floor is marked by folding of the sediments, with overturned beds just below the fault plane. There is a zone about 20 m wide, 960 m below the sea floor, containing fragmented and sheared rock. Below it, the rocks are virtually undeformed. This zone we identify as the décollement. The complete interval between the thrust and the décollement shows pervasively deformed sediments, with abundant minor faulting at depth, whereas complex shear bands predominate

near the thrust fault. There is evidence that shear bands pre-date the faulting. Thus we have demonstrated that the style of sediment deformation changes with time and/or position within the accretionary prism.

The physical properties of the recovered sediments were measured at about 1-m intervals throughout the cored section. The most remarkable discovery here was

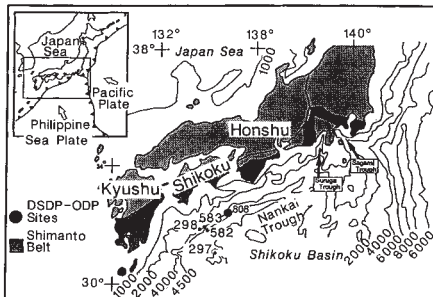


FIG. 2 Location map of site 808, Nankai Trough.

the large contrast in properties occurring across the décollement. For example, there is a considerable increase in porosity below the décollement, violating the normal pattern by which porosity decreases with depth owing to compaction. This may indicate that the sediments contain interstitial pore water at higher than normal (hydrostatic) pressures.

The mode of pore water expulsion from accreted sediments, whether localized along fault zones or diffused through the sediments, may affect the style of structural deformation in the prism. Our chemical analyses show that the composition of the interstitial water changes both with the changing lithology and at the two major faults. A large smooth decrease in chlorinity towards the décollement can be interpreted either as evidence of fossil fluid flow or as a result of an *in situ* reaction. But there is no direct evidence of active localized fluid flow. This contrasts with results from the Barbados accretionary prism, drilled previously^{4,5}, where there is strong evidence of significant recent fluid flow along the décollement and other faults.

We also made measurements of physi-

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cal properties in the boreholes themselves, but only in limited sections of the holes drilled, owing to unstable hole conditions. The thermal gradient at 120 °C km⁻¹ is steep, and combined with measurements of thermal conductivity indicates that the heat flux locally is 130 mW m⁻². An analysis of hydrocarbon gases from the hole suggests that the thermal gradient may have been even higher in the past.

The most important single result of this leg is the quantification of the marked contrast in structure and physical properties across the décollement, which occurs despite the lithological uniformity of the rocks. There is abundant evidence of subhorizontal shortening in the accreted sediments and virtually no deformation in

the underthrust sediments. Furthermore, the large offset in physical properties suggests a considerable difference in the physical conditions of stress and pore pressure above and below. Despite these differences, interstitial water geochemistry showed no chemical signature of concentrated active fluid flow. The complete absence of mineralized veins, or of any firm evidence of fluid flow, suggests that the hydrogeology of at least this section of the Nankai prism is radically different from the Barbados prism. □

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CELL MOTILITY

Dynamin joins the family

Peter J. Hollenbeck

IN our attempt to understand the evolution of protein function, analogy sometimes fails us. Consider two classes of proteins in particular, the mechanochemical enzymes and the GTP-binding proteins: although both classes undergo a cycle of nucleotide binding, hydrolysis and release, their relatedness has commonly been considered to end at that single shared property. After all, mechanochemical enzymes couple their nucleotide cycle to the generation of force for movement, whereas GTP-binding proteins are generally thought to couple GTP binding and hydrolysis to regulation of activity of other proteins. But work reported by Obar *et al.* on page 256 of this issue¹ now provides evidence that one mechanochemical enzyme, dynamin, belongs to a family of GTP-binding proteins, and raises the possibility that both functional and evolutionary relationships exist between proteins whose behaviour is modulated by GTP-binding, and those which generate force for movement.

Dynamin is a recently discovered mechanochemical ATPase that mediates sliding between microtubules². It comprises a polypeptide of relative molecular mass 100,000 (known as D100) which is sufficient to bundle microtubules in an ATP-sensitive fashion, and an unidentified cofactor which is required for the hydrolysis of ATP and for sliding motility. Obar and colleagues obtained and sequenced a complementary DNA clone encoding the entire D100 polypeptide, and they demonstrate that it contains not only a consensus ATP-binding site, but also two additional elements indicative of GTP binding, the sequence and spacing of which are conserved among GTP-binding proteins. Moreover, there is strong sequence similarity between D100 and two related GTP-binding proteins — the

Mx proteins³ and the product of the yeast vacuolar protein sorting gene, *VPS1* (ref. 4). Obar *et al.* argue that this homology shows that dynamin and the Mx and VPS1 proteins are members of a distinct family of GTP-binding proteins. They go on to consider how a GTP-binding protein can be a mechanochemical enzyme.

Members of the superfamily of GTP-binding proteins have a common function — the development of increased affinity for a target when they bind GTP, and loss of that affinity when GTP is hydrolysed — that arose early in evolution and that has been adapted to a variety of purposes. Different families of GTP-binding proteins can be classified according to what events are regulated by their cycle of GTP binding and hydrolysis: for the G-protein⁵ and *ras* protein⁶ families, it is signal transduction and amplification; for the tubulins⁷, polymerization and depolymerization; and for the initiation and elongation factors of protein synthesis⁸, it is the unidirectional transport of aminoacyl-transfer RNAs to the ribosome. For a number of GTP-binding proteins involved in the transport of proteins into the endoplasmic reticulum^{9–11}, and thence to and through the Golgi towards their eventual secretion^{12–14}, the function of the GTP cycle is less clear. Bourne¹⁵ suggests that these proteins are transport GTPases that drive the vectorial transport of protein-containing vesicles from station to station along the exocytic pathway. In this model, the membrane compartments of the exocytic pathway contain different marker proteins, and the transport GTPases couple the binding and hydrolysis of GTP to the loading of material at proximal markers, and unloading at distal ones.

For dynamin and its GTP-binding cousins, Obar *et al.* take the case a step further. A cycle of GTP binding and

hydrolysis coupled not to regulatory interactions, but to transport, sounds rather like the force-generating cross-bridge cycle of mechanochemical enzymes. Thus, based upon both analogy of function and homology of sequence, Obar *et al.* propose that VPS1, and perhaps other secretion factors, actually serve as mechanochemical GTPases during transport, generating microtubule-based vesicle movement coupled to the binding and hydrolysis of GTP. This model differs from that of Bourne in that the information for the direction of transport could reside not only in markers on the membrane compartments, but also in the inherent polarity of microtubules and in the order in which the various membrane compartments array themselves along the microtubules. Dynamin itself would then be viewed as a member of this family specialized for microtubule–microtubule, rather than microtubule–vesicle interactions, and Obar *et al.* point out unique features of the D100 sequence that are consistent with this interpretation.

Is a system of mechanochemical GTPases consistent with our current view of intracellular transport? ATP, rather than GTP, is believed to be the physiological substrate for most mechanochemical enzymes, but it is noteworthy that all of them (including dynamin) can bind and hydrolyse GTP, and at least one — the microtubule-based motor, kinesin — can use GTP to produce movement¹⁶. In this context, Obar and colleagues point out that kinesin's primary sequence contains, in addition to an ATP-binding element, one of the two additional elements associated with GTP binding. This raises an interesting question. Could some mechanochemical ATPases be derived from the ancient and venerable family of GTP-binding proteins — descendants that have lost their preference for GTP, along with their ancestral function, in the ATP-rich environment of the eukaryotic cell? □

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Seeing is believing

John E. Hearst

A new microscope described elsewhere in this issue by G. J. Puppels *et al.* (*Nature* **347**, 301–303; 1990) represents a milestone in the long history of cytology. The instrument can focus on a volume of less than $1\ \mu\text{m}^3$ (a volume approaching the theoretical diffraction limit of a microscope using light of wavelength 660 nm), and obtain a high-quality Raman spectrum. Because Raman-scattering spectra are sensitive to molecular vibration, such a spectrum allows one to 'see' chemical bonds, and therefore to distinguish between protein and nucleic acid.

As the technique becomes more completely developed and more rigorously applied, it could be used to explore far more subtle structural and compositional variations than are possible at the resolution of the conventional light microscope. As a new arrow in the quiver of procedures available to cytologists for determination of biological structure, confocal Raman microspectroscopy is an exceptional development.

The authors have used a combination of emerging technologies in a manner never before applied to microspectrophotometry. First, a charge-coupled device (CCD) is used as the photon detector. CCDs have been used primarily in astronomy because of their extremely high signal sensitivity achieved at low background intensities. Second, to achieve these low backgrounds, a chevron-type dielectric band-pass filter tuned to suppress the Rayleigh (or elastic) scattering of the incident 660-nm laser light with an efficiency of one part in 10^9 is used. Finally, the confocal optics allow for the tight localization of the detected inelastically scattered light to the $1\ \mu\text{m}^3$ -volume element. The authors have determined the changes in the ratio of DNA to protein in salivary chromosome bands, interbands and telomeres, demonstrating the extraordinary capabilities of Raman microspectroscopy.

Genetics has had a special relationship with cytology since the 1870s, when light microscopy first revealed the existence of chromosomes. Ever since then, the quality of cytological representations of chromosomes has always outclassed molecular understanding. The cytological maps of *Drosophila* salivary chromosomes pioneered by Bridges, for example, established the relationship between genetic linkage and microscopically observable bands and distances. This work preceded the observations in 1944 by Avery and his co-workers that established DNA as the genetic carrier. In the 1960s, Joseph Gall developed the technique of *in situ* hybridiza-

tion, by which many of the simple satellite DNA sequences, discovered by density gradient sedimentation and renaturation kinetics, were shown to be structurally unique to the chromosome, localizing at centromeres and telomeres. There is a major distinction between heterochromatin and euchromatin, a cytological density difference inferred to be related to biological activity. The banding patterns of salivary chromosomes and correlations between the numbers of bands and the numbers of genes in *Drosophila* make these issues even more intriguing.

The discovery of banding patterns in human chromosomes and their importance to karyotyping introduced the question again. What are the compositional and structural properties of the chromosome that create these observable cytological features, and why do they exist? Although tremendous in-

ONCOGENIC VIRUSES

Of marmots and men

Don Ganem

ONE of the great unsolved problems in the biology of human cancer is the reason for the close association of hepatocellular carcinoma (HCC) with chronic infection by hepatitis B virus (HBV). This link is no small matter: over 250 million humans are chronic carriers of HBV, and in the vast areas where HBV infection is endemic, HCC ranks among the most common human tumours. On page 294 of this issue, Fourel *et al.*¹ shed new light on this connection by showing that integrated viral DNA is often linked with known oncogenes in an animal model of liver cancer.

The fact that HBV carriers are between 100 and 200 times more likely than non-carriers to contract HCC is compelling epidemiological evidence for a link between HBV and HCC². Almost all HBV-related HCCs harbour integrated viral DNA, usually in multiple copies. Tumours are clonal with respect to these insertions, indicating that integration of viral DNA precedes or accompanies the transforming event.

Further evidence of a link comes from animal studies. Although HBV infection is largely restricted to humans, related viruses affect a number of other species, including woodchucks (marmots), ground squirrels and ducks. Remarkably, woodchucks infected from birth with woodchuck hepatitis virus (WHV) are almost guaranteed to develop HCC³.

The oncogenic sword of HBV is terrible

roads have been made at the level of DNA sequence, nucleosome structure and, to a lesser degree, chromatin coiling, we remain remarkably inept at mapping the compositional and structural properties of larger domains. Confocal Raman microspectroscopy is a most promising technique for alleviating our difficulties, for it provides *in situ* compositional and structural information without the use of invasive stains and fixatives that are common features of most cytological and microfluorometric analyses.

What remains to be seen is what else can be studied by this technique, for there are potential limitations associated with contrast and resolution. If the $1\text{-}\mu\text{m}^3$ volume contains too complex a mixture of chemically distinct molecules, assignment of Raman lines may be impossible. On the other hand, the sensitivity of this instrument is remarkable. □

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but not swift: tumours typically develop only after 20–30 years of persistent infection, and are usually accompanied by signs of hepatocyte necrosis and active inflammation (chronic active hepatitis) and fibroblastic proliferation (cirrhosis). Hepatoma induction by WHV is similar to that by HBV, except that tumours develop earlier in the course of infection and at a much higher frequency.

The molecular mechanisms underlying the connection between HBV and HCC have been elusive. The long incubation period of HCC argues against the existence of a dominant oncogene encoded by the viral genome, and most experiments designed to find one have been unsuccessful. In the absence of such a gene, most models for how HBV engenders HCC fall into two broad categories, according to whether viral DNA contributes directly or indirectly to hepatocellular proliferation. In the past year, evidence in support of both views has accumulated.

Models in which hepadnaviruses (the class of DNA virus that includes HBV) make a direct genetic contribution to the loss of growth control are influenced by studies of retroviruses (such as murine leukaemia virus, MuLV) that lack dominant oncogenes. These viruses often activate cellular proto-oncogenes by the integration of viral sequences in the flanking host DNA. But efforts to identify such oncogenes in the vicinity of integrated

HBV genomes in human HCC have generally been unsuccessful, although individual examples of HBV insertions next to genes for retinoic acid receptor⁴ and cyclin A⁵ have been identified. Unfortunately, these account for only a tiny fraction of human hepatomas. In this context, the findings of Fourel *et al.*¹ on WHV-related HCC present a striking contrast: of 30 hepatomas in woodchucks carrying WHV, six have insertions of WHV DNA adjacent to cellular *N-myc* sequences, and 18 of the tumours overexpress *N-myc* messenger RNA, normally undetectable in woodchuck liver. Additional woodchuck hepatomas previously reported by this group harbour WHV insertions near the related *c-myc* locus⁶.

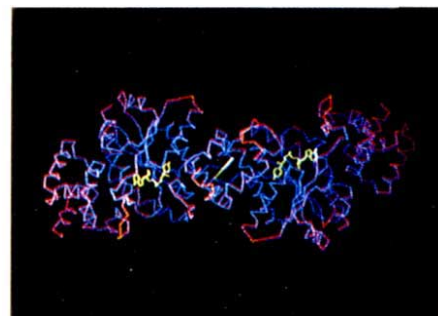
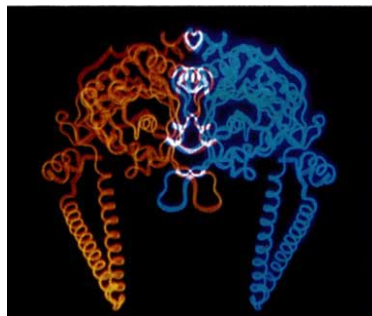
N-myc genes were initially discovered by virtue of their amplification in human neuroblastoma⁷: more recently, insertion of MuLV DNA adjacent to *N-myc* has been noted in MuLV-induced T-cell lymphomas, in which it is also associated with enhanced accumulation of *N-myc* RNA⁸. The activation of *N-myc* expression in woodchuck HCC is similar to that by MuLV: in both cases, insertion of viral sequences occurs in the 3'-noncoding region of the *N-myc* locus and in the same transcriptional orientation, leading to chimaeric RNA. Potent viral enhancers are thereby linked to the *N-myc* locus, as are signals for 3' end processing. This affords both transcriptional and post-transcriptional opportunities for the upregulation of *N-myc* messenger RNA levels; which of these predominate in WHV-related HCCs remains to be determined.

Interestingly, woodchucks possess two *N-myc* loci. One seems to be the product of an ancestral reverse transcription event and most WHV insertions involve this locus. The preservation of the coding function of this gene suggests that it plays a part in normal growth and development, the nature of which remains unknown.

Besides the activation of known oncogenes, various other genetic events in flanking host DNA, such as deletions and translocations, have been associated with the integration of viral DNA⁹: these, too, could contribute directly to growth deregulation. And beyond these pathways of insertional mutagenesis are still other ways in which HBVs could influence proliferation directly. In particular, mutated or deregulated viral gene products might do so by activating important cellular functions *in trans* (this might explain those cases in which *N-myc* expression was increased in the absence of a nearby WHV insertion). The viral X-gene product is a transcriptional activator able to augment expression from many cellular and viral promoters. Although chromosomal integrations often disrupt the X coding region near its 3' end, many such truncated products retain transactivation activity (ref. 10 and K. Matsubara, personal communication).

Synthetase with a difference

THE genetic code lies at the heart of molecular biology, and central to the code are the aminoacyl-transfer RNA synthetases that charge tRNAs with the appropriate amino acid. The structures of the charging enzymes determine the genetic code by their combined specificity for binding particular tRNAs and amino acids. The report by



David Blow

S. Cusack *et al.* on page 249 of this issue describes the fourth crystal structure of an aminoacyl-tRNA synthetase (seryl tRNA synthetase, at left above). Remarkably this structure bears no resemblance to the three aminoacyl-tRNA synthetase structures previously determined (for example that of tyrosyl tRNA synthetase, right), and thus suggests a deep division in the genetic code.

The three aminoacyl-tRNA structures previously known all share a common structural motif — the dinucleotide-binding fold that interacts with the ATP that drives the charging reaction. It had been speculated that all the charging enzymes would have this fold and thus share a common origin in the distant evolutionary past. The new results with the sequence analyses of G. Eriani *et al.* in last week's issue (*Nature* 347, 203–206; 1990), show that this is not so, and that aminoacyl-tRNA synthetases fall into at least two classes with no sign of any evolutionary relationship. The significance of this division is an outstanding puzzle in the evolution of the genetic code. G. N.

munication). The potential role of the X protein in hepatocyte proliferation has recently been underscored by the observation of HCC induction in two lines of transgenic mice that overexpress the viral X gene (C. Kim and G. Jay, personal communication).

The frequent rearrangements of viral DNA that accompany or follow integration can also generate novel products with the potential to alter the programme of gene expression. A remarkable example is the truncated envelope glycoprotein encoded by an HBV integrant cloned from a human HCC by Kekule *et al.*¹¹. In cotransfection experiments, this protein can enhance reporter gene expression. Because the protein is both glycosylated and secreted (U. Lauer and A. Kekule, personal communication), it might act by activating transmembrane signalling pathways, though other interpretations are possible. But there is as yet no direct evidence linking the activity of this protein with cell proliferation.

A second class of explanations for the HBV-HCC connection proceeds from a different set of observations. Pathologists have long known that whereas the link between HCC and chronic HBV infection is particularly strong, hepatoma develops at a lesser but still elevated rate in other conditions characterized by long-standing hepatocellular injury, such as alcoholic cirrhosis, α_1 antitrypsin deficiency and haemochromatosis. What all these conditions have in common is that hepatocyte

necrosis triggers a stereotypical host response marked by chronic inflammation, fibrosis and (importantly) hepatocyte regeneration. It is presumed that this regenerative hyperplasia expands the population of cells at risk of subsequent genetic changes (mutations, chromosomal rearrangements and so on) that further deregulate cell growth. According to this view, the contribution of HBV to oncogenesis is indirect — evoking the host immune responses that lead to the destruction of infected cells and trigger this programmed response.

An important experiment¹² nicely illustrates this progression. Chisari *et al.* constructed transgenic mice that disproportionately express one of the three HBV envelope glycoproteins. This disruption of the normal stoichiometry of these proteins leads to the retention of all three in the endoplasmic reticulum in nonsecretable aggregates, the accumulation of which ultimately kills the cell. By 2–3 months of age, these mice develop liver necrosis and inflammation, with consequent reactive hyperplasia in the ensuing months. Ultimately, in transgenic lines with the greatest degrees of glycoprotein retention, HCC follows in nearly all animals. Although envelope protein dysregulation may or may not be an important cause of cellular injury in human hepatitis B (the alternative being cell lysis by virus-induced cytotoxic T cells), the potential for viral infection to set in motion this chain of events is clear. ►

Can these divergent views of the contribution of HBV to oncogenesis be reconciled? There is no reason why both direct and indirect mechanisms cannot operate *in vivo*. The fact that liver injury of any cause invariably provokes hepatocyte proliferation means that even in the absence of a specific genetic contribution from the viral genome, there is likely to be a background of transforming events not directly linked to viral DNA. For proponents of direct models, the challenge now is to define to what extent subsets of human HCC exist in which integrated HBV sequences act to drive proliferation over and above this background, and to clarify the mechanisms by which this occurs. Clues to such cases might emerge from consideration of the differences between WHV- and HBV- associated oncogenesis, chief among which is the earlier onset of hepatoma during chronic WHV infection. Perhaps the subset of human HCCs that develop earlier than usual in the carrier

state would be enriched for such integrants. The finding of frequent oncogene activation in woodchuck HCC will surely invigorate the search for comparable events in human hepatomas. □

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GEOPHYSICS

Enhancing mantle conductivity

L. M. Hirsch

THE cause of a layer of high electrical conductivity in the Earth's upper mantle¹ at depths in the region of 40–180 km has long intrigued and puzzled geophysicists. Both above and below the layer, conductivity profiles can be reconciled with laboratory conductivities of mantle minerals under controlled conditions^{2,3}. Conversely, conductivities in the high-conductivity layer are about 1–2 orders of magnitude greater than would be reasonably expected from solid-state conduction in the volumetrically most important minerals. Small amounts of good conductors such as partial melt⁴, water⁵ or carbon⁶ are often invoked to explain this anomaly. The common approach is to treat the layer as a mixed medium containing insulating silicate crystals mixed with an interconnected fluid or grain-boundary phase of high conductivity. On page 272 of this issue⁷, Karato presents a new possibility: hydrogen dissolved in the olivine lattice may substantially increase the electrical conductivity of the host crystal. Thus, enhanced conduction outside mineral grains would not be necessary.

Karato bases his idea on diffusivity measurements of hydrogen⁷, assuming that the hydrogen contributes as charged ions, presumably protons, to electrical conduction in olivine. Because hydrogen is mobile in the [100] crystallographic direction of olivine, only a modest amount of hydrogen — H/Si content between about 200–2,000 parts per million — would be sufficient to account for the high-conductivity layer. Although direct evidence of such hydrogen concentrations

within olivines and their fluid inclusions from most mantle-derived xenoliths is limited⁸, this circumstance may simply be caused by hydrogen loss during ascent because of its rapid diffusivity in olivine⁹.

Many mechanical and petrological stability problems are avoided with Karato's hydrogen hypothesis because the hydrogen is intracrystalline and the amount of hydrogen required to enhance conductivity is below that which would cause extensive partial melting⁶. In contrast, with the water or melt hypotheses, one is left with the problem of explaining how the mechanical stability of an interconnected fluid phase can be maintained over long distances in the upper mantle for long geological times⁴. And the carbon explanation is constrained by the temperature and pressure for diamond stability, the grain-boundary diffusivity of carbon, and oxidation state of the upper mantle⁷.

To verify Karato's hypothesis, several issues need to be resolved. First, it must be experimentally demonstrated that hydrogen exists as a charged defect in olivine at high temperatures and pressures. Second, we need estimates of the hydrogen content of the upper mantle and the relative partitioning of hydrogen among co-existing mantle minerals. Third, we require a chemical rationale that explains why enhanced conductivity by hydrogen does not persist beyond the high conductivity layer at both shallower and deeper depths. Finally, we need to evaluate what influence the considerable anisotropy of hydrogen diffusivity in oli-

vine, spanning two orders of magnitude⁷, may have on the amount of hydrogen required for enhanced conduction. For isotropic mixtures, this anisotropy would increase by a factor of only about 3 Karato's values for the hydrogen content. But significant texture of olivine in the region of the high-conductivity layer could produce a more profound effect on hydrogen-enhanced electrical conductivity.

The cause of the high-conductivity layer is a critical question in geophysics. This layer is located in a region of the upper mantle that has several remarkable features that are intimately connected with the thermal and rheological state of the Earth's mantle and crust. Beneath the rigid lithospheric plates (70 km thick on average) there is the asthenosphere, a more plastic layer that permits vertical isostatic adjustments of land masses and lateral shear to occur. This shear is driven by convective motions in the mantle and has a direct bearing on the motion of the plates and surface deformations. Another feature is the low-velocity zone — an effective decrease in seismic-wave velocity that starts within a depth range of about 50 to 220 km. Many geophysicists argue that partial melting gives a comprehensive explanation for the low-velocity zone, high-conductivity layer and the weakness of the asthenosphere. The mechanisms responsible for each of these features, however, could differ: both the high-conductivity layer and the low-velocity zone are often absent beneath continental shields whereas isostatic adjustments from the last ice age, such as in the Canadian and Baltic shields, indicate the presence of the asthenosphere. Yet, although there is no necessary connection between high conductivity and asthenospheric plasticity, hydrogen concentrations of the order 100–3,000 p.p.m., such as invoked by Karato, may also weaken the upper mantle by a factor of 1.5–3 (refs 9,10).

Of course, high electrical conductivity can have many causes. We may find that high conductivity layers in different localities in fact have different origins. □

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Rotons put physics in a whirl

P. V. E. McClintock

WHAT is a roton? The existence of rotons, the particle-like excitations that carry much of the thermal energy of superfluid helium-4, has been thoroughly substantiated through numerous experiments and much is known about their properties. Yet the physical nature of the roton remains almost as obscure as when its existence was first proposed by Landau¹ half a century ago. But a little progress is now being made towards a resolution of this question

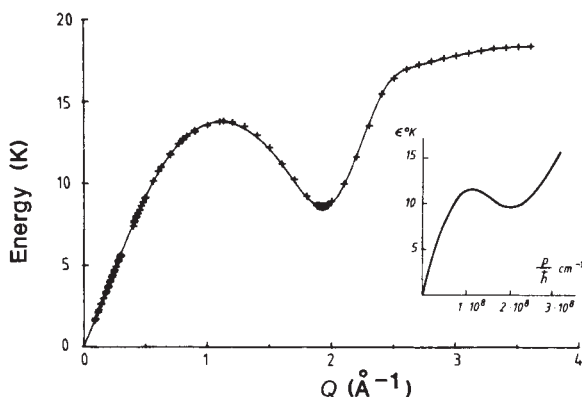


FIG. 1 The experimental dispersion curve for the excitations in superfluid helium-4 under its saturated vapour pressure in the low-temperature limit. Their energy (divided by Boltzmann's constant k) is plotted as ordinate against their momenta (divided by Planck's constant $\hbar$) as abscissa. The excitations near the origin are phonons; those near the local minimum are known as maxons; and those near the local minimum are called rotons. (From ref. 5 based on data from ref. 6. Inset is Landau's prediction.)

and it formed one of the main topics at a recent conference* on quantum fluids.

Rotons are just one species in the bestiary of collective excitations found in condensed matter. Like phonons, the vibrational excitations of a crystal lattice, or photons, the excitations of the electromagnetic field, these quanta of superfluid helium-4 behave like particles insofar as they carry measurable momentum and energy. Indeed, their best characterized aspect is undoubtedly their dispersion relation, connecting these quantities.

This can be measured by scattering beams of neutrons off the superfluid helium: any change in the neutron's momentum and energy must be attributable to the creation or destruction of one or more excitations. The compiled data (Fig. 1) make a curve remarkably similar to Landau's inspired guess (inset on Fig. 1) which he based on early measurements of superfluid helium's specific heat capacity. Rotons are the excitations that lie close to the local minimum in the curve. The other excitations portrayed, very different in character, are phonons in the linear region at low energy, and

'maxons' close to the local maximum.

In the roton region, the dispersion curve takes on a parabolic form, just as one gets for a free particle moving in a vacuum. But there is an important difference: the minimum of the curve comes at a finite momentum rather than at zero momentum. A roton at the minimum consequently has the curious property of remaining stationary in the liquid (the speed, or 'group' velocity, of an excitation being determined by the gradient of a tangent to the dispersion curve), despite its large momentum. Rotons just to the left of the minimum which, even more astonishingly, move backwards in the opposite direction to their momenta, are known as R^- rotons; those on the high-momentum side are called R^+ rotons.

It has long been held that a roton is probably surrounded by a dipolar velocity field so that the superfluid streams around it in a pattern similar to the distribution of magnetic field round a small bar magnet. R.J. Donnelly (University of Oregon) has now used this idea to calculate how rotons are scattered by quantized vortices (the quanta of rotation) in superfluid helium-4 (Fig. 2). It can be seen that rotons colliding with the left of a vortex with clockwise circulation are only marginally deflected; whereas those to the right suffer an almost total reversal of direction, undergoing a species change from R^+ to R^- rotons. This approach yields values of the so-called mutual friction parameters that are in good agreement with those values derived from thermal transport measurements.

Although the dipolar velocity field provides a good picture of the roton when viewed from afar, it gives little in the way of clues as to the nature of the enigmatic entity at its centre. G.A. Williams (University of California at Los Angeles) reminded the meeting that Feynman's intuitively appealing picture² of the roton as a microscope vortex ring would yield the right kind of flow field although, according to Roberts and Jones' calculations³ of a few years ago, it is apparently not correct in its original form. More experimental evidence is needed.

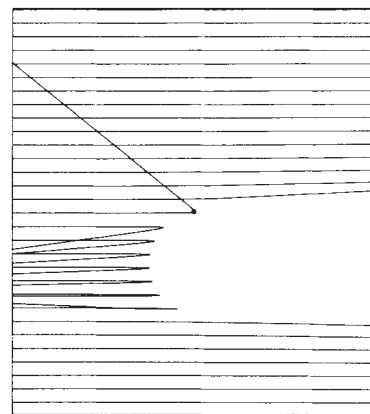


FIG. 2 Calculated trajectories of rotons incident upon a vortex line (indicated by the dot) with clockwise circulation. (From ref. 7.)

One of the few ways of obtaining information about the roton core, borrowing a technique from particle physics, is to study the interactions of colliding beams of rotons. Making a pair of crossed-beams of rotons by pulse heating a tank of superfluid helium-4 with two thin-film contacts, and detecting them via the free atoms they eject into the vacuum above the superfluid's surface, A.F.G. Wyatt (University of Exeter) and colleagues discovered that the scattering cross-section is remarkably momentum dependent (Fig. 3).

The origin of this intriguing effect is still controversial, Donnelly and I. Iwamoto (University of Tokyo) offering competing explanations. But given that the cross-section for beams of featureless hard spheres is constant — the experiments must be telling us something quite fundamental about the nature of the roton.

The new information coming from recent neutron scattering experiments is complementary to such studies. It amounts to much more than just the mapping out of the low-temperature dispersion curve of Fig. 1, which is merely the trace of the maxima of scattering peaks. The intrinsic width and structure of a particular scattering peak is replete with detailed information. The temperature

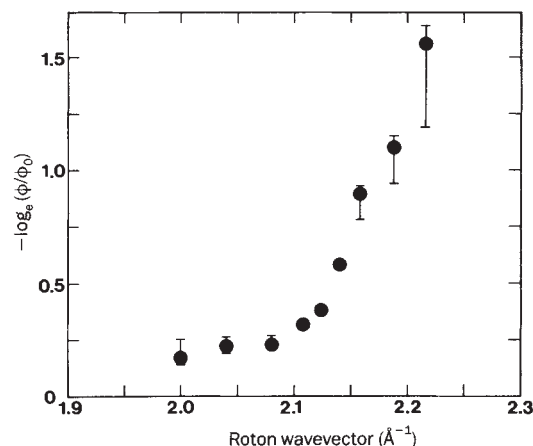


FIG. 3 The attenuation (proportional to the roton-roton cross-section) of two beams of rotons colliding at right angles, measured as a function of the momenta of rotons in one of the beams. (From ref. 8.)

* NATO Advanced Research Workshop Excitations in 2D and 3D Quantum Fluids, Exeter, 10–15 August 1990.

dependence of the scattering intensity is of particular interest in that, for certain parameter ranges (such as the maxon and roton regions) it consists of two principal features: a sharp peak superimposed upon a broader background (Fig. 4). It is evident that the sharp peak disappears above the transition temperature, T_λ , at which the superfluid turns normal.

Considerable debate centred on the physical significance of the relative intensity of the two peaks in Fig. 4. A widely accepted perception, clearly articulated by A. Griffin (University of Toronto), is that the phonons on one hand, and the rotons and maxons on the other, are inherently different kinds of excitation — quite to the contrary of what one might assume from the continuous character of the dispersion curve in Fig. 1. Phonons, being density fluctuations of the whole fluid, are detected by neutron scattering both above and below T_λ . Rotons and maxons, on the other hand, are more localized 'single-particle' excitations of some (unspecified) kind, and are probably characterized by an entirely distinct dispersion curve above T_λ at which

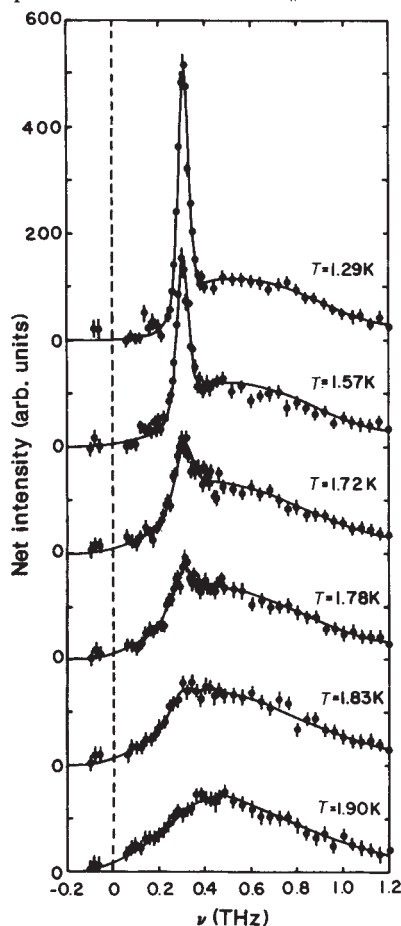


FIG. 4 The neutron scattering intensity from superfluid helium at the maxon, plotted as a function of energy (divided by Planck's constant $\hbar$) for several temperatures. For the ambient pressure of 20 bar, the superfluid transition temperature $T_\lambda = 1.93$ K. It is evident that the sharp peak disappears at T_λ . (From ref. 9.)

temperature they cannot, unfortunately, be detected by neutron scattering. Below T_λ , the existence of a small fraction n_0 of the helium atoms occupying the zero-momentum 'Bose-condensed' state has profound theoretical consequences. In particular, it causes the phonon and roton/maxon branches of the dispersion curve to combine, through a quantum-mechanical process known as hybridization, thereby forming the single dispersion curve observed in the experiments. Correspondingly, the maxon/roton excitations then become detectable by neutron scattering (the sharp peak evident in Fig. 4) with a weight that is proportional to the fraction n_0 .

This argument depends, of course, on the existence of a non-zero condensate fraction (which should be carefully distinguished from the superfluid fraction, representing the proportion of the fluid which displays superfluid properties and which approaches 100 per cent below 1 K). Fortunately, n_0 itself can also be measured by inelastic neutron scattering, albeit in collisions with a much higher transfer of momentum. P. E. Sokol (Pennsylvania State University) was thereby able to derive a value of $n_0 \sim 0.10$ as $T \rightarrow 0$, agreeing within experimental error with earlier neutron results by Svensson and co-workers⁷ based on a rather different approach. This is of course very much a finite value and it is also consistent with recent theoretical predictions.

Given that rotons crop up in all facets of superfluid helium-4 research — light-scattering studies, diffusion of helium-3 through the superfluid, and the crystallization of solid helium-4, for example, all discussed at the meeting — it is remarkable that so little is known of their physical character. But then, after 50 years of study, superfluid helium-4 remains in many ways much more mysterious than its younger relatives superfluid helium-3 and superconductivity. The prospect held out at the meeting is that we should soon come to grips with what makes it so much more complex. □

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Deep digestion

MOST land animals use the nitrogen in their food proteins very inefficiently. They excrete it as urea or uric acid, making no attempt to burn these waste products usefully. Yet urea and uric acid are quite feasible fuels. As manufacturers of high explosives know, an oxidative decomposition which releases free nitrogen gas also releases a lot of energy. Why don't animals exploit this energy?

The reason, says Daedalus, is that nitrogen is almost insoluble in water. Carbon dioxide, our main gaseous waste product, is soluble enough to be transported round the body in solution. But a creature that generated nitrogen internally would find itself in trouble. It would swell up with bubbles of undissolved gas, and might even burst.

Divers risk a similar fate. Under the high pressure of the depths, the nitrogen in the air they breathe dissolves in their body fluids. When they come back to normal pressures it comes out again, and may form bubbles in the blood or synovial fluid. This is the painful and dangerous diving syndrome, 'the bends'.

So, says Daedalus, the ultimate metabolic efficiency should be shown by deep-sea creatures, living under pressures high enough for nitrogen to be easily soluble in water. Freed from solubility problems, they could metabolize proteins and other nitrogenous foods completely to nitrogen, perhaps feasting on the steady fall of nitrogenous wastes from the low-pressure creatures far above them.

DREADCO's biologists are now studying deep-sea lantern-fish, worms and sponges in special pressurized aquaria and culture vessels, looking for novel enzymes that metabolize urea and other nitrogenous wastes. If they find them, they will look for traces of them in land creatures too. A small amount of nitrogen metabolism, not enough to cause the bends but giving a little added efficiency while slightly shrinking the excretion load, could well have evolved in many species. Selective breeding, or even genetic engineering, could develop it further.

At first Daedalus dreamt of breeding a special non-excreting urban dog, which metabolized its food all the way down to nitrogen gas; but he soon realized that at atmospheric pressure it would rapidly succumb to the bends. He then recalled his previous scheme for a high pressure hen house or turkey coop, to revive the flight instincts of these sedate birds. So he now plans to develop a breed of non-excreting high-efficiency chickens, turning nearly all their food into meat and eggs with minimum waste and mess. Their special coop will be pressurized with oxygen and argon, and tended by farm labourers in diving gear.

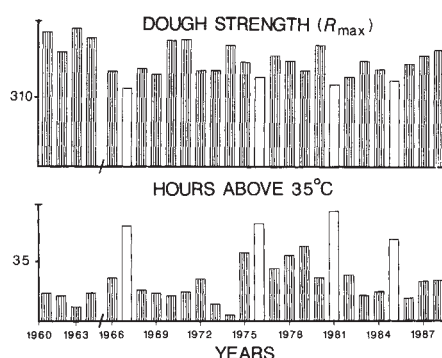
David Jones

Global warming and wheat

SIR—In the 'greenhouse' debate, little attention has been paid to the impact of rising temperatures on the quality characteristics of crops which, particularly for wheat and barley, affect their suitability for use and therefore market values.

High growth temperatures are important factors in wheat-growing countries such as Australia, where temperatures during grain-filling often rise well above the moderate (say 30 °C) temperatures traditional for cereal growing. Such temperature fluctuations (say more than 35 °C for only a day or two) could account for previously unexplained changes in crop quality, according to our research.

Our survey¹ of Australian prime hard wheat quality during the past decade (see figure) indicates that the seasons when



Weaker dough strength for Australian prime hard wheat coincides with high-temperature episodes. During the past decades, those seasons when dough strength (R_{max}) was least were the ones with the hottest ripening periods (hours above 35 °C at Narrabri from 1 October to 12 December).

dough strength was at its lowest (irrespective of changing variety mix) occurred in those years with the greatest number of hours over 35 °C during the grain-filling period. Retrospective study of wheat-variety trials verifies this trend: 'hot' seasons were associated with weaker dough properties (and thus poorer breadmaking quality) for all but one variety. Possibly some such genotypes will provide genetic sources of tolerance to heat.

We have performed some experiments to explain (and possibly counteract) the dough-weakening effect of high growth temperatures. Wheat seedlings synthesize heat-shock proteins in response to an hour's exposure to 40 °C of heat. Some of these proteins are similar to the range produced in other organisms, but we detected one characteristic 14-amino-acid peptide in coleoptiles and roots.

Twelve of these residues are identical to the 12 amino-terminal residues of α/β gliadins, previously found exclusively in the endosperm of wheat grain. Furthermore, the location of the gene encoding this heat-induced peptide (on the short

arm of chromosome 6D) is the same as that encoding α/β -gliadin².

Examination of published nucleotide sequences of cereal-storage proteins reveal the presence of five heat-shock elements (the sequence responsible for switching on heat-shock proteins at high temperature) only in the leader sequence of α/β -gliadin. Increased synthesis of gliadins (part of the wheat-gluten complex) would account for the dough-weakening effect of high temperatures. That gliadin synthesis does increase under such circumstances has been demonstrated with heads in liquid culture. Furthermore, grain exposed to high temperatures in the glasshouse and the field has more gliadin and a weaker dough property than normal.

If greater temperature fluctuations occur, as predicted by the greenhouse model, periods of high temperatures during the grain-filling period of temperate cereals are likely to increase in both frequency and severity. Our results may suggest a method for the genetic modification of temperate cereals to meet the challenge of increasing global temperatures.

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Radioactive dust

SIR—In his recent Scientific Correspondence¹, Taisei Nomura said that "some studies at Sellafield have revealed an unusually high contamination of workers' homes with radioactive materials". The reference quoted by Nomura was an editorial by Beral² who used essentially the same phrase indicated above but referenced our work³. In that study, we identified one radionuclide of possible workplace origin and commented on its minor dosimetric significance.

Our study, conducted between 1984 and 1986, was designed to identify any enhanced levels of radioactivity in domestic dust and to assess its radiological significance. It encompassed 14 communities in west Cumbria in the north of England; we sampled about 170 houses including 25 each in Seascale and Ravenglass. We found various radionuclides of Sellafield origin; alpha-emitting nuclides contributed most to committed effective dose equivalent (CEDE).

We concluded that estimated CEDE

values were small in relation to natural background radiation and represented a very low level of risk. In two houses in Seascale, we found elevated concentrations of the gamma-emitting nuclide ⁶⁰Co, probably of workplace origin. But the dosimetric consequences of these levels was small compared with that referred to above from other radionuclides; for the ingestion pathway approximately 7% of the total for an adult and 1% for a 1-year-old child. There is evidence, therefore, that, over the period of our study, contamination of workers' homes was of minor dosimetric significance compared with the general indoor exposure of Sellafield origin and that this in turn constituted a very low level of risk.

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Ozone depletion

SIR—In a recent NASA/WMO report¹, it was suggested that a 2–3% decrease in total ozone occurred in the mid-latitudes of the Northern Hemisphere during 1969–86. Depletions of up to 6% may occur in both hemispheres in future depending on man's ability to limit emissions of certain halogen-containing trace gases. But the development of the ozone hole in the stratosphere over Antarctica during winter^{2,3} has more serious short-term implications. This ozone depletion, of up to 75%, could be due to reactions involving chlorofluorocarbons⁴.

Atkinson and Eason reported⁵ record low total ozone concentrations over southern Australia during December 1987. In particular, they found a reduction of 25 Dobson units (DU) in Perth on 11 December and a 35 DU decrease in Melbourne between 11 and 13 December, similar with effects in Hobart and Lauder, New Zealand. These low values were thought to be due to the transport of ozone-depleted air from the Antarctic⁶.

The Australian Radiation Laboratory has broadband solar ultraviolet radiation detectors at 14 locations throughout the country and at the three permanently manned Australian stations in Antarctica. But for December 1987 only Melbourne data were available, comprising spectral measurements on cloud-free days and continuous broadband measurements. We have analysed these data to gauge the impact the ozone depletion and the subsequent increase in solar ultraviolet radiation may have had on exposed people.

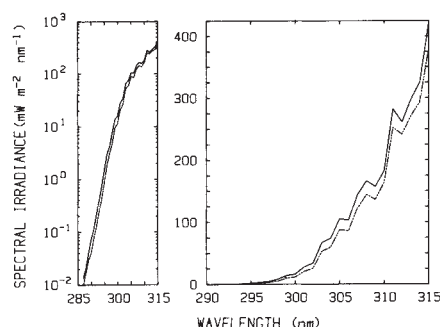


FIG. 1 Solar ultraviolet B spectral power distribution for 10 and 14 December 1987. (Broken and solid lines, respectively.) Measurements for Melbourne under cloud-free conditions.

We used a microcomputer-controlled spectroradiometer incorporating a double-grating monochromator⁷ to measure global ultraviolet radiation incident on a horizontal plane. The broadband measurement system comprises four radiometers (solar radiation, total ultraviolet, ultraviolet B and actinic B) and acquires data continuously. Figure 1 shows the solar ultraviolet B spectral power distributions obtained at solar noon on 10 and 14 December — that is, before and after the event described by Atkinson *et al.*⁶. The percentage increase in the spectral irradiance for 14 December over that for 10 December as a result of the ozone decrease was 50% (at approximately 297 nm), 25% (at 303 nm) and effectively 0% (at 325 nm). Such variation results from the rapid decrease in the ozone absorption coefficient across this wavelength region.

Significantly, ozone levels dropped on 10 December and remained low until 19 January 1988. Figure 2 shows the solar noon ultraviolet B irradiances, obtained by integrating the spectral irradiances over 285–315 nm. The broadband ultraviolet B detector gave a similar profile and as expected, there is a negative correlation between these data and the midday ozone column.

Between 10 and 14 December, the 10.5% decrease in ozone concentration

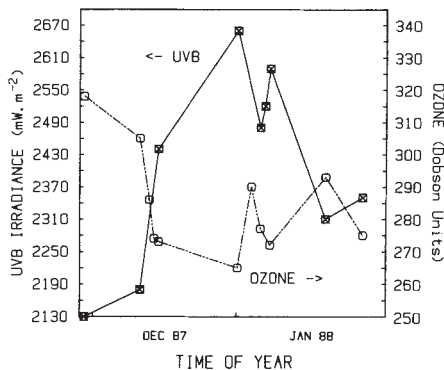


FIG. 2 Comparison of solar ultraviolet B irradiance and total ozone for several days during December 1987 and January 1988. Ozone data provided by R. Atkinson of the Australian Bureau of Meteorology.

resulted in an 11.9% increase in ultraviolet B irradiance and about a 21% increase in the effective irradiance⁸. Hence, at this latitude, the percentage increase in effective ultraviolet B irradiance for a 1% decrease in ozone, is about two.

There is concern over whether this episode of ozone depletion will have harmful effects such as an increase in the already high rates of skin cancer. But because of increased cloud cover, total ambient ultraviolet B radiation for December 1987/January 1988 was no greater than that of December 1988/January 1989, even though higher irradiances were detected on the cloud-free days of the 1987/1988 period.

We conclude that ozone depletion occurring in Antarctica is already having an effect on populated regions of the Southern Hemisphere as a result of the transport of air after the spring break-up

of the Antarctic vortex. The effect may be greater in future if an ozone depletion period were to coincide with an extended cloud-free period.

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Martian electron spectra explained

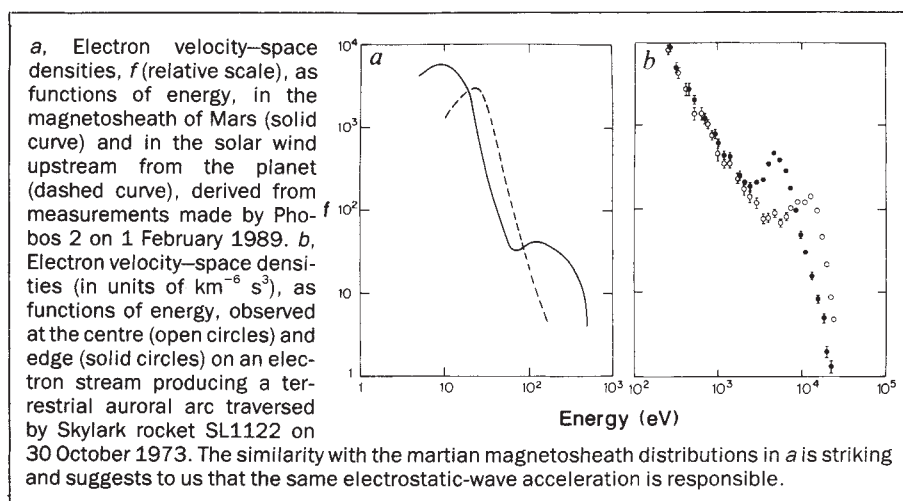
SIR — Last year it was reported by N. M. S. *et al.*¹ that when the Soviet spacecraft Phobos approached Mars on 12 February 1989 and entered the magnetosheath of heated solar wind behind the bow shock upstream from the planet, the energy distribution of electrons was generally characterized by two distinct peaks. We have been studying the energization of electrons in the aurora and other space plasmas, and would like to suggest an explanation for this observation, based on an energy-selective acceleration of solar-wind electrons by electrostatic waves at the martian bow shock. If our explanation is correct, this would add further weight to the growing evidence for the universality of electrostatic-wave acceleration of electrons in space plasmas.

Figure 4 of ref. 1 shows an electron spectrum (count rate as a function of energy) observed in the martian magnetosheath at 18:36 UT. This spectrum exhibits two peaks. In our figure (a) we translate the spectrum into a distribution of relative velocity-space densities, f , by dividing count rates by the square of kinetic energy. In a dynamical system (not the case here), f will remain unchanged, even though the energy at which a given value occurs may vary (Liouville's theorem). The solar-wind distribution, observed 31 min earlier at 18:05 UT, and from which the magnetosheath distribution is derived, is added for comparison. Acceleration at the bow shock has introduced the new features of a minimum in f at 70 eV, a peak at 115 eV and a high-energy tail, such major distortions signifying a non-dynamical process in operation. The process may also have caused the reduction in f , or equivalently the reduction in energy at a given f , below 70 eV. All these features, and the modifications they

imply, are natural consequences of a stochastic acceleration process favouring electrons in a selected band of energies, as some of us have recently demonstrated by numerical simulation² and a deterministic approach^{2,3} to the problem.

The stochastic exchange of energy and momentum between electrons and packets of electrostatic waves of the lower-hybrid type is such a process. Electrons and wave packets can interact only when the phase and group velocities of the waves match those of the electrons. Although the wave fronts propagate relatively slowly and almost perpendicular to the magnetic vector, $\mathbf{B}$, their projections parallel to $\mathbf{B}$ have velocities comparable to those of the electrons which, being strongly magnetized in the space plasmas under consideration, are constrained to flow in this direction. This resonance permits momentum and energy to be exchanged, the sense and rate of flow depending on the velocity distributions of the electrons and wave packets. Net flow is from waves to electrons when the electron distribution has a negative slope, as in the solar wind (a in the figure). Energy selection is expected because the process is so effective at low parallel velocities, where electron densities are highest, that the growth of waves is suppressed. A high-velocity limit can also be anticipated.

The concurrent observation that plasma waves near the shock appear as broadband noise extending from below the lower-hybrid frequency to the electron plasma frequency⁴, is a positive, but not conclusive, indication of the presence of lower-hybrid waves in the region of acceleration. It remains to be seen whether the waves were of sufficiently high intensity. The free energy for generation of the waves derives in this interpretation



from the streaming energy of the solar-wind protons observed⁵ from Phobos 2 to be retarded and heated at a location reached at 18:25 UT, intermediate between the observations of single- and double-peaked spectra.

Our explanation, that the lower peak is an unchanged property of the original solar-wind distribution and the higher peak is due to acceleration by lower-hybrid waves, is a direct application of a mechanism we have proposed^{2,3,6-8} to account for electron acceleration in the terrestrial aurora, where very similar electron distributions can be observed (**b** in the figure), and in other instances of electron energization in space plasmas. The mechanism is of practical value in experimental nuclear fusion devices where the magnetic field of the current carried by electrons accelerated by lower-hybrid

waves helps to contain the plasma, and electron energy communicated to ions serves to heat the plasma⁹.

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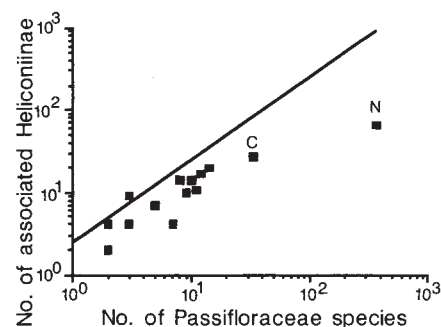
Fewer species

SIR—Erwin¹ obtained 1,200 species of beetle from the canopies of just 19 individuals of one tropical tree species, causing him to suggest that there could be 30 million arthropod species in the whole of the tropics, rather than 1.5 million, the approximate number of species then thought to exist. Stork² evaluated Erwin's assumptions and produced a range of values from 7 to 80 million or more species. But I calculate tropical arthropod diversity to be about 6–9 million species.

To produce their estimates, Erwin and Stork had to estimate the proportion of insect species specific to a given tree species. These authors ignored other insects as transients because these insects should be counted as genuine associates of other plants. But this categorization of insects is rather simplistic³. Many herbivores feeding on a given tropical plant species also feed on other plants in the same community³, and insects are often specialists on different plant species in different places. If these insects are categorized as specific, they will be counted more than once in estimates of global diversity because the

extrapolation procedure relies on multiplying the number of specific herbivores per plant by the number of plant species.

After eliminating transients, I corrected for the overlap in herbivore faunas between plants. A parameter (x) allows the number of herbivore species supported by a plant community (a) to be



Relationship between the number of Passifloraceae species recorded and the number of Heliconiinae species associated with those plants. Unlabelled points show data for single sites (from refs 4, 5, 8). C, combined data from Rincon, Arima Valley, Rio de Janeiro and Corcovado sites; N, total for neotropics (data from refs 6, 7); line, number of Heliconiinae species expected if a linear projection is made on the basis of the average number of Heliconiinae (2.73) on single *Passiflora* populations.

estimated from the number of species of herbivore per plant population (b) by the equation $a = x \times b \times c$, where c is the number of plant species in the community. For well-known communities, x can be calculated. For the herbivores of passion vines (*Passiflora*) at a site in Costa Rica (Sirena)³, there are 30 herbivores for which adequate data exist (a), an average of 11.7 herbivore species per plant (b) and 9 *Passiflora* species (c), so x is 0.28. For the relatively host-specific Heliconiinae butterflies on *Passiflora*, estimates of x for four communities are 0.56 for the Arima Valley, Trinidad, 0.71 for Rincon, Costa Rica, 0.29 for Rio de Janeiro and 0.56 for Corcovado (including Sirena) in Costa Rica^{4,5}. The mean x is 0.45. Without this correction, multiplying the number of plant species by the number of herbivores per plant produces, an estimate of community diversity that is too high by a factor of about two ($1/0.45 = 2.2$).

A parameter (y) that might allow the estimation of approximate global (or continental) diversity (d) from the number of species of herbivore per plant population (b) is given by $d = y \times b \times e$, where e is the world (or continental) number of plant species. Again, this parameter can be calculated for well-known groups. There are about 66 species of neotropical Heliconiinae butterflies⁶, and a minimum estimate of the number of Passifloraceae in the American continents is 360 species⁷. From studies of 45 Passifloraceae populations drawn from 33 species^{4,5}, the average number of species of Heliconiinae per Passifloraceae population is 2.73. These values give an estimated y of 0.067. Without this correction, extrapolation from the number of Heliconiinae species on one Passifloraceae population to those associated with all American Passifloraceae would result in an overestimate by a factor of 15 ($1/0.067$; see figure). Tentatively, the overestimate can be partitioned into a within-community component of about 2, and a between-community component of about 7 ($15/2.2$).

Using $y = 0.067$ as a correction factor, I have recalculated diversity from Erwin's data and assumptions, except that I assume beetles to be 20% rather than 40% of tropical arthropod species². This gives a value of 3 million arthropod species in tropical canopies, or 6–9 million if ground arthropods² are included.

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Horizontal flow of pore fluids

SIR—Hanor, in his calculation¹ of the density-driven subsurface movement of pore fluids near a salt dome on the Louisiana Gulf Coast, derived horizontal velocities of the order of 10 m per year at depths exceeding 1,000 m. These velocities are large for a sedimentary basin — Bethke *et al.*² cite values measured in cm per year — even in the geologically disturbed environs of a salt dome. Indeed, they are gross overestimates which stem from the erroneous assumption of hydrostatic equilibrium in the fluid flow. We repeated Hanor's calculations using a model that does not invoke the assumption of hydrostatic equilibrium and obtained velocities smaller by a factor of thousands.

Although the assumption of hydrostatic equilibrium may lead to reasonable approximations of the absolute values of pressure, it is entirely inappropriate in predicting horizontal pressure gradients (horizontal components of "hydraulic force field" in Hanor's terminology) and the associated velocities of lateral density-driven flow in deep basins. Indeed, the hydrostatic assumption (also sometimes referred to as the Dupuit approximation) is tantamount to the neglect of the resistance to vertical fluid flow or, equivalently, to the assumption that the permeability is relatively large in the vertical direction (comparable to or greater than the permeability in the horizontal direction). This assumption is not valid for the geological system under consideration.

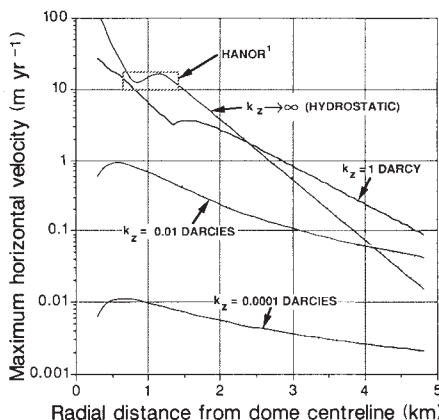
In reality, the vertical permeability in deep sedimentary basins, evaluated over thick sequences of alternating sands and shales, is not large, even next to a salt dome, as Hanor himself has more recently implied³. Moreover, convective fluid overturn, which is typical of density-driven fluid movement⁴, will almost always be accompanied by a significant component of vertical flow, especially near an upward-tilting boundary such as a dome. Such a vertical flow will cause the system to deviate markedly from hydrostatic equilibrium.

In our calculations, we used a model of density-driven flow that combines the equation of continuity (conservation of mass) with Darcy's law for flow through porous media⁵. The model is not based on the hydrostatic assumption, and, moreover, recognizes that subsurface systems characteristically display highly anisotropic permeability behaviour when taken over thick sand/shale sequences, featuring very different values for permeability in the horizontal and vertical directions.

We sought to match as closely as possible Hanor's input parameters, including the imposed density distribution, the shape of the salt dome and values of all key physical properties. We used Hanor's value of 10^{-12} m^2 (1 darcy) for the hori-

zontal permeability but varied the vertical permeability in the calculations. We also extended the calculations farther from the dome, using the reasonable constraint that the fluid density perturbation associated with the salt dome dies out gradually with horizontal distance.

As expected, our results agree with those of Hanor when the vertical permeability is set to a relatively large value,



A comparison of our results with Hanor's data from ref. 1.

of the order of 1 darcy or greater (see figure). But with more realistic values of the vertical permeability between 10^{-16} and 10^{-17} m^2 (0.00001–0.0001 darcy), recently implied by Hanor as being reasonable for regions adjacent to salt domes, our calculated velocities decrease to between 0.1 and 1.0 cm per year. The calculations also show that the flow disturbance caused by density variations diminishes markedly with distance from the salt dome, indicating that this phenomenon is localized.

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HANOR REPLIES—Miller *et al.* misrepresent the purpose of my calculations¹ which were made to demonstrate that inversions in pore-water density arising from variations in temperature, pressure and salinity are of sufficient magnitude to drive kilometre-scale vertical overturn of subsurface pore waters at the Iberia salt dome, Louisiana Gulf Coast. But the calculations of Miller *et al.* support my hypothesis. The limitations of the approximation I used were completely understood and are specifically mentioned in my paper. Further, subsequent published calculations by me and others^{3,5} using more rigorous techniques and more realistic boundary conditions have yielded

comparable estimates for the range of the magnitude of hydraulic force which can be generated by spatial variations in pore-water salinity and temperature around salt domes.

Contrary to the impression that may be given by Miller *et al.*, I did not attempt to determine the fluid velocity field at Iberia because of the lack of adequate field information. To provide a preliminary order-of-magnitude estimate of fluid velocities which could result from the calculated forces, however, I did show that a horizontal hydraulic force of 50 Pa m^{-1} operating on fluids contained within a sand having an intrinsic permeability of 10^{-12} m^2 could induce velocities in excess of 10 m yr^{-1} . This choice of permeability was an appropriate reference value because massive sands at the Iberia dome are known from direct measurement to have such permeabilities and because Iberia is surrounded by a sand-dominated, not a clay-dominated sequence (see my Fig. 3). My more recent work⁶ shows that even clay layers in the Louisiana Gulf coast can have effective vertical permeabilities two or three orders of magnitude higher than the values cited by Miller *et al.* as "more realistic", supposedly on my authority.

The detailed understanding of rates of deep fluid flow in the Gulf Coast will require more information than is generally available at present on the nature and local variability of driving forces and on the effects of media heterogeneity on permeability. The oversimplified conceptual and numerical modelling approach employed by Miller *et al.* is inadequate. Their conclusion, "... that the flow disturbance caused by density variations diminishes very markedly with radial distance from the salt dome, indicating that this phenomenon is very localized", is contradicted by published geological field evidence^{7,8} clearly demonstrating that salt-dome-related solute transport is regional, not local. If Miller and his colleagues want to determine rates of subsurface fluid flow and solute transport in Louisiana, I recommend they begin monitoring the migration of the 7×10^3 litres of liquid hazardous wastes injected into the south Louisiana salt-dome province by their employer.

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Less than total recall

Stuart Sutherland

Human Memory: Theory and Practice. By Alan Baddeley. *Erlbaum: 1990. Pp. 515. Hbk £25, \$39.95; pbk £11.95, \$19.95.*

A RECENT dictionary defines "Boxology" as "The construction and ostentatious display of meaningless flow diagrams as a substitute for thought". *Human Memory* contains some splendid examples. One has sixteen arrows connecting thirteen boxes, which are given such labels as encoding, recoding, conversion, cognitive environment, original engram and recollective experience. Even so, the author admits, the diagram is "a simplified version of the original". Just how the processes subsumed in each box are carried out, we are not told.

The main point of the diagram is that if anyone wants to recall material presented on a particular occasion he needs not only to remember the material but to have associated it with that occasion. Everyone already knows the word 'cold', but not everyone dragooned into being a subject in a psychologist's laboratory will recall that it was one of the fifty words read out to him there. Indeed, as Endel Tulving showed in a famous recent experiment on memory, the subject may fail even to recognize a word as part of the ensemble presented if given the wrong cue for retrieval, while being able to recall it if given an appropriate reminder of the occasion. (This finding was thought remarkable because normally recognition is much superior to recall.) The principle that one can remember what happened on a given occasion only if the events have been associated with it may well strike readers as rather obvious, but apparently it came as a surprise to workers in memory: it has been given the grandiose title "synergistic memory theory".

Baddeley does a competent job of expounding such experiments. He lays considerable stress on what he calls "theories", though for the most part they are vague speculations little better than dressed-up common sense. Work on human memory seems to make little progress, although this is not for want of effort, if one counts effort in man-hours rather than in intellectual exertion. There is still no clear answer to that old examination chestnut "Why do we forget?" Is it that the traces laid down in the nervous system (presumably changes in connectivity at synapses) decay spontaneously with time? Is it because material learned more recently changes the connections made in previous learning and hence makes it harder to retrieve the original material? Or do new traces fade more quickly than old ones so that if the mater-

ial to be learned is similar to that already learned, the earlier material comes to overshadow the later? Are memories preserved only as a result of calling them to mind periodically? Or was Freud right in thinking that anything unacceptable to the ego is repressed and hence unavailable at a conscious level?

We do not know whether spontaneous decay of memory traces occurs, but we have known for many years that forgetting is increased by learning similar material either before or after that on which a person is tested. Baddeley is sceptical about repression, but points out that people asked to remember events from their early life remember a greater number of pleasant than unpleasant ones — but he fails to note that perhaps there really are more pleasant than unpleasant events. Not surprisingly, there is a tendency to recall more sad events when one is unhappy than when happy, and to colour one's interpretation of previous events with the current sadness. Oddly, there is little or no change in the number of happy events recalled depending on one's mood, though one may doubt how far laboratory manipulations really succeed in inducing either sadness or happiness.

Baddeley has curiously little to say about one of the most interesting recent

discoveries in memory research. Explicit memory is the deliberate recollection of an experience, whereas implicit memory is the influencing of a response by a previous experience without the person knowing that he is being influenced. For example, if someone has recently read a list of words including the term 'clock', and is then asked to fill in the missing letters in 'clo--', he is much more likely to respond to 'clock' than 'close' even though he has no conscious recollection of having seen the word 'clock'. Such implicit memory differs from explicit memory in several ways, some of which are as follows. Changing the modality in which the word is presented, from, for example, vision to hearing, has no effect on explicit memory but decreases implicit memory. Asking subjects to process words at a high level, for example by thinking of their meaning rather than just their sound, improves explicit memory, but has no effect on implicit memory. The rate at which words are forgotten is very different (in rather complex ways) for the two kinds of memory. Learning new but similar material impairs explicit but not implicit memory. The two kinds of memory appear to be entirely separate because there is almost no correlation between success on the same item in an implicit and explicit memory task. Although Baddeley ignores these striking results, which are relevant to consciousness, he does mention that many amnesics with little or no explicit memory have comparatively intact implicit memory.

One unusual feature in what is basically a textbook on memory is the account of the application of work on learning to the



Land of the giants — early European travellers came back with over-awed and exaggerated descriptions of tropical rain forests; the engraving above (originally from *Flora Brasiliensis*, 1840) shows an artist's impression of a scene in the Atlantic coastal forest of Brazil and is reproduced from *An Introduction to Tropical Rain Forests* by T. C. Whitmore. This book describes the rain forests, their structure and dynamics, flora and fauna, and discusses their value to mankind and our impact on them. With examples taken from all parts of the humid tropics, the book is aimed at the general reader. Published by Oxford University Press, price is £35 (hbk), £16.95 (pbk).

treatment of the mentally disordered. This section is, however, skimmed and as Baddeley himself remarks "the conclusions that I draw . . . are, therefore, inevitably superficial". A second innovation is a chapter on treatment for amnesic patients, but the results are meagre. Persuading these people to associate names with visual imagery helps them remember the names, while their memory for the substance of a passage of text can be improved by the PQRS technique (preview the material; question to find the salient points; read carefully; state the main features of the text; and finally test oneself by checking memory for the text against it). Both techniques are of course known to help unaffected people a little, although they are based on common sense rather than psychological findings.

Although lacking in excitement, the book is for the most part competently written, provided you can bear the repeated misuse of the expression "as such". The same cannot be said for the index, which is a disgrace. The contents are not memorable: only readers adept at the PQRS technique are likely to commit them to mind. □

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Forcing the tissue

R. H. T. Edwards

Skeletal Muscle in Health and Disease. A Textbook of Muscle Physiology. By D. A. Jones and J. M. Round. *Manchester University Press: 1990. Pp. 221. Hbk £29.95, \$49.95. Pbk £9.95, \$17.95.*

FOR hundreds of years, skeletal muscle has presented an intriguing challenge to those interested in understanding the generation of force and other physiological and metabolic properties of this tissue. The principal models that are the basis of our understanding of skeletal muscle structure, function and chemistry are largely derived from studies of muscle isolated from various animals. Over the past 20 years, the growth of methods of chemical analyses of needle biopsy samples and electrical stimulation techniques has been quite remarkable, allowing human muscle to be studied to something approaching a degree of precision similar to work on isolated muscle. The authors of this book have distilled in their short volume a wealth of experience in developing and applying techniques for the study of human muscle in health and disease.

The strength of the didactic account of the subject given here lies in the fact that both authors are laboratory researchers who have worked in clinical departments, with the result that discussions on basic mechanisms are extended to man by, for example, descriptions of a cross-section of muscle obtained by X-ray computerized tomography and a wealth of (admittedly monochrome) photomicrographs of muscle in various pathological states. The account of muscle structure and function is greatly helped by illustrations of the structure and inter-relations of particular cellular components. Classical muscle physiology is concisely but authoritatively covered with a profusion of specific examples on different isolated muscle preparations.

The growth and development of muscle and the implications for athletic performance lead on to an up-to-date account of modern concepts of physical training. This account is particularly interesting in view of the authors' own observations on the relationship between cross-sectional area measured by X-ray tomography and the increase in maximal isometric strength with training. Force has long been known to be proportional to cross-sectional area of muscle, but it has not been at all clear how muscles can become stronger with little or no increase in cross-sectional area. The authors suggest that tethering by new connective tissue increases force through

a reduction in effective fibre length. Also of interest to athletic performance is the section on the adaptations for endurance exercise, which covers well-documented work carried out in Scandinavia, the United Kingdom and in North America on the energy supply processes for muscular activity.

The sections on fatigue and pain are those in which the authors make their most original contributions. Their account of the complexities of the mechanisms underlying fatigue in isolated muscle and in human subjects is commendably clear and concise. Vigorous muscular activity is always at risk of causing muscle damage or pain, and it is refreshing to see the systematic analysis of the mechanisms underlying these based on experimental observations.

In the final section, the authors consider the reactions of muscle not only to mechanical damage but also to the consequences of disease. Muscle as a specialized tissue responds to insult in relatively few ways. What is seen as compatible with a particular clinical diagnosis comprises both primary features and (often more obviously) secondary adaptations to damage or compensatory use. Jones and Round provide examples of these adaptive responses obtained from biopsies from people with Duchenne muscular dystrophy, spinal muscular atrophy and inflammatory muscle diseases.

The value of percutaneous muscle biopsy as a tool to give a 'life vision' (*Bios, Opsis*) to supplement the physiological and metabolic study of human muscle disease is obvious. The recent, rapid increase in our knowledge of the muscular dystrophies obtained from molecular-biology methods is summarized here for Duchenne and Becker muscular dystrophies, together with reference to the possible physiological role of the cytoskeletal protein dystrophin which is absent in people with these types of muscular dystrophy.

This text is likely to become a standard for student courses on cell biology, physiology, sports sciences and research methods in physiotherapy. The book deserves a wider readership, however, as an exciting introduction to the rapidly expanding field of muscle biology and how it has influenced our understanding of human muscle performance and the pathophysiology of muscle disease. □

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■ Some of the techniques mentioned above are covered in *Scanning Electron Microscopy, X-Ray Microanalysis, and Analytical Electron Microscopy*, new from Plenum. This laboratory workbook, by Charles E. Lyman *et al.*, provides exercises for students developed at courses held annually for the past few years at Lehigh University. Spiral bound, price is \$29.95. □

Monod and stereo

John Galloway

Mechanisms of Cooperativity and Allosteric Regulation in Proteins. By Max Perutz. Cambridge University Press: 1990. Pp. 101. Pbk £11.95, \$17.95.

'ALLOSTERY' does not feature in Macmillan's *Dictionary of the History of Science* — a rather striking omission. But Ptolemy's spheres do. Possibly with a touch of irony, Max Perutz likens the appeal of symmetry for Jacques Monod, who formulated the idea of allostery, with the aesthetic attraction exercised by the spheres. Thus Perutz links second-century astronomy with twentieth-century molecular biology through one of the principles that threads its way through all science. It turns out that symmetry is not necessarily the central issue in allostery (although it is sometimes important) any more than helicity is necessarily the central issue in the structure of DNA, although these issues are the features most people know about in each case.

I mention allostery and DNA together because if DNA was nature's first secret, allostery was certainly its second, as Monod claimed. Neither could be called a secret today. Perutz's book describes how for allostery the word has been made flesh using the inexorable methodology of X-ray crystallography. Be warned though, the 'book' is actually a 100-page article reprinted from *Quarterly Reviews of Biophysics*, and will be tough going for most readers. Take with a small pinch of salt the blurb's claim that the book may "appeal to anyone wanting to understand the interplay between chemistry and motion that is the very essence of life". It may, but you will have to work at it.

What exactly is allostery? Some proteins are now known to have two three-dimensional structures, and an allosteric transition is the switch between one structure and the other. Monod, and those with whom he worked closely on the idea of allostery in the early 1960s, were interested in the idea of metabolic interconnectedness — that critical metabolic steps might (must) be controlled by "metabolites acting as physiological signals rather than as chemically necessary components of the reaction itself". The systems exercising such control seemed very diverse, but could they be explained by a few simple general principles?

Monod and his collaborators proposed that the control was mediated by proteins with special properties. In general, they thought these proteins possessed two receptor sites — an active one responsible for the protein's actual biological activity and binding the substrate, and an allosteric site more or less remote from the

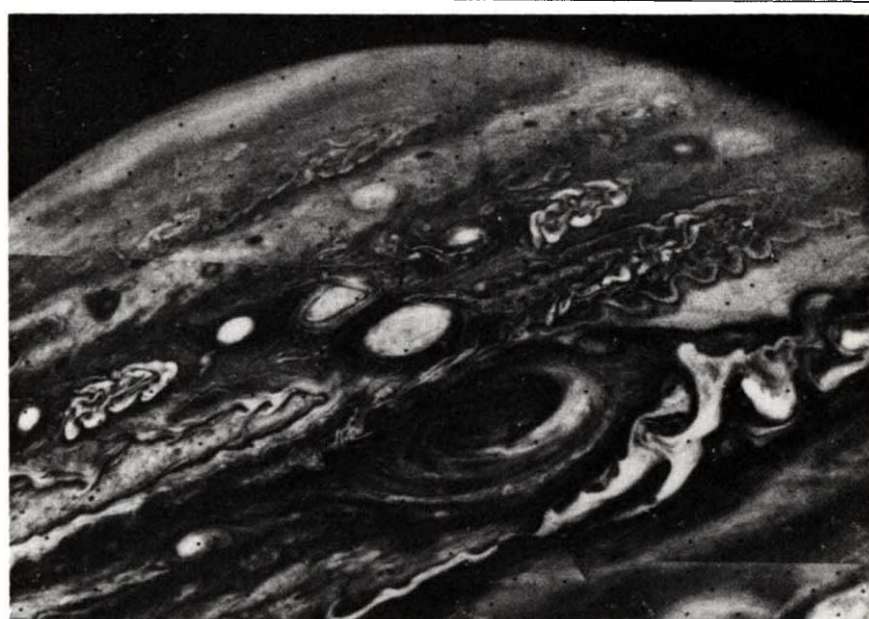
active site and binding an effector molecule, a binding that caused a (reversible) change in the protein structure and altered the properties of the active site. More particularly, Monod *et al.* suggested that the most interesting instances of allostery would involve cooperativity between identical sites. Cooperativity seemed to demand some sort of repetitiveness in the protein's structure, in other words a symmetrical arrangement of identical subunits (or an elaboration on such a theme). Transition in the tertiary structure of the subunits would be translated into a corresponding transition in the quaternary structure of the whole molecule between an inactive state in which the subunits were tightly bound — which Monod *et al.* called T (for tense) — and an active state, R (for relaxed). Taken to extremes, a relaxed state is a state of dissociation of the protein into its individual subunits.

Perutz's review was stimulated by the spate of enzyme and other protein structures that were published in 1988 in which both R and T structures were described. Perutz describes the structures of the molecules and the way they work in great detail, the sort of detail we now expect crystallography to provide. The predictions of Monod *et al.* are brilliantly borne out. "The subunit contacts of haemoglobin, phosphofructokinase and aspartate transcarbamylase, are very clearly designed to allow only concerted transitions between alternative quaternary structures".

Today we know very well what presumably was not known in the early 1960s — that proteins combine elements of both rigidity and flexibility in their structures,

both exploited in the biological roles they play. Looking back to those early days it is striking how structural theory was running ahead of experiment. Although the Monod school was interested in enzymes, its seminal paper, published in 1965 in the *Journal of Molecular Biology*, actually opens with a discussion of haemoglobin. Perutz in his new book praises the boldness and imagination of "an analogy between feedback inhibition in aspartate transcarbamylase and threonine deaminase and inhibition of haemoglobin by hydrogen ions" which led to the paper. And indeed the first third of his review describes haemoglobin and its close molecular relations. Interestingly, Perutz's own papers do not seem to refer to Monod's idea before 1970, when he had obtained good high-resolution data on both forms of the molecule. He wrote in *Nature* that year "For purposes of comparison, haemoglobin can be compared to an enzyme made up of two different pairs of subunits with oxygen as the substrate, haem as the enzyme and H⁺ and DPG as allosteric effectors".

The interplay between rigidity and flexibility in proteins makes it tempting to draw a comparison with the theory of the structure of the spherical viruses. In the years leading up to Monod and collaborators' first papers on allostery in the early 1960s, Aaron Klug and Don Caspar were propounding their theory of the structure of the spherical viruses based on Buckminster Fuller's geodesic domes. The theory included the necessity of quasi-equivalence — seemingly identical proteins being arranged non-equivalently in the viral coat and demanding flexibility



"To boldly go" — a mosaic of Voyager 1 images of Jupiter, taken in violet light. The Great Red Spot can be clearly seen, with violet eddying around it. *The Journeys of Voyager: NASA Reaches For The Planets* by Robin Kerrod is a pictorial record of the Voyager encounters with the planets and their moons. Stunning colour photographs help tell the whole Voyager tale from the beginning. Published by Prion, price is £9.95. □

in the way they bonded to one another.

In 1964, electron micrographs of human-wart, simian 40 and polyoma viruses were published, showing pretty convincingly that this theory was correct. In a report in *Science* that year by Victor McElheny, Perutz was quoted as saying "The results had surprised us all. The theory was pretty far fetched, you know." Why far fetched? The obvious answer is that protein crystallographers did not think that protein structures could be flexible and hence provide a mechanism.

It does seem an extraordinary coincidence, looking back, that theory and experiment were running on such parallel tracks. Of course what really matters is how luminous an idea is, and by any

standards allostery is pretty luminous. An elegant idea shows how the self-regulation of very complicated systems is possible. Whether Monod's school thought that its idea could be realized only through its specific model is not clear — at least to me. What Perutz's review brings home is the power of crystallography to show how the idea is realised in concrete terms. Detailed descriptions free us to some extent from the reliance by theory on metaphor or analogy and over simple preconceptions. Whether the descriptive richness is yet quite the same thing as a full understanding remains to be seen. □

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Watching brief

Jeremy J. D. Greenwood

Waterfowl Ecology. By Myrlyn Owen and Jeffrey M. Black. Blackie: 1990. Pp.194. Hbk £30, pbk £13.95. Published in the United States by Chapman and Hall. Hbk \$71.50, pbk \$35.

Ducks, geese and swans — the avian family Anatidae — are the particular favourites of many birdwatchers. Most of them are relatively large and live on water or in open terrestrial habitats where they are easy to observe, often in large numbers. Furthermore, they are easy to tame and they appear to behave fairly normally when rendered flightless by wing-clipping, so they make good subjects for both private collections and public displays. There can be few children in Europe and North America who have not fed the ducks or swans on their local pond. Wildfowl also make good quarry, which not only means that there are many hunters with a passionate interest in them but that they are the basis of a huge industry serving those hunters. Others shoot — or otherwise seek to control — some species (especially geese) because of the damage they do, or appear to do, to crops. And since the perceptions of birdwatchers, wildfowlers and pest-controllers may be different, these birds and their ecology are often the subject of controversy.

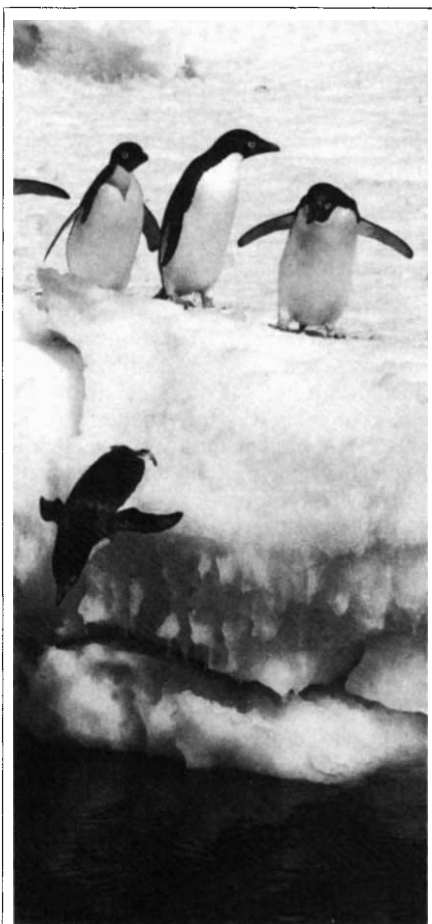
So a book on wildfowl ecology should be useful. One written by Myrlyn Owen and Jeffrey Black, of the Wildfowl and Wetlands Trust, ought to be good. This result is both useful and good. It covers not only the whole of wildfowl ecology in the strict sense, with chapters on food and population dynamics, but also other aspects of the birds' natural history (breeding biology, social behaviour and movements). It does so systematically and in relation to general evolutionary and

ecological principles. It is up-to-date in outlook and content, and lively to read. Because the family comprises a limited number of fairly similar species, the authors have been able to be comprehensive without being tedious or overwhelming; yet there is sufficient diversity to provide useful contrasts.

Blackie's Tertiary Level Biology Series, to which this book belongs, is designed for specialist courses at the advanced undergraduate level. Although there are unlikely to be many such courses devoted exclusively to wildfowl, the popularity of the group is such that this book will provide useful background reading for broader courses. Students will find here a wealth of solid examples, from species with which they may be very familiar, to illustrate the general principles that they have learned from more general texts or courses in ecology, wildlife management or ornithology. The book will also be a useful tool for the education of new graduate students embarking on wildfowl research, for academic ecologists working on other organisms who wish to broaden their knowledge, and for wildlife managers who wish to know more about a group of birds to which so much management effort has been devoted.

Yet *Waterfowl Ecology* left me feeling disappointed on two counts. Although the material is presented firmly in a framework of general principles, these principles themselves are rarely brought into clear focus. The book is certainly not 'ecology, as illustrated by waterfowl'. But then it shouldn't be: the reader should know the principles before coming to this book. My disappointment was therefore unjustified. Nonetheless, I have a lingering feeling that the chapter on population dynamics should have had a full population model for at least one species and certainly a mention of key factor analysis.

My second disappointment came at the end of the chapter on conservation and management. Given the immense experi-



Taking the plunge — Adelie penguins are bold and curious birds, often unable to resist the temptation to follow should one of their colleagues stroll along the beach or dive into the sea. Every October the penguins return to their rookeries around the coasts of the Antarctic, sometimes walking or tobogganing over as much as 96 km of ice. *Birds in Focus* by Mark Cawardine is filled with more than 200 superb photos and many fascinating facts about birds of all kinds. Published by Salamander Books, price is £16.95. □

ence of wildfowl management, I wished that the chapter had been twice as long. But that would have unbalanced what is now a well-balanced book and the authors were right to keep each part of their subject within bounds.

Wildfowl provide some of the finest birdwatching memories: the crooning of courting eiders, the wild calls of newly arrived whooper swans passing over a Scottish firth at night, the sky-music of a distant skein of barnacle geese moving over snowy mountains, the clamour of pinkfeet tumbling out of the sky to their roosting water, and the delight of small children at the ducklings on the village pond. Owen and Black provide a substantial contribution to an intellectual appreciation that will complement such aesthetic experiences. □

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Computer modelling of solid-state inorganic materials

C. R. A. Catlow & Geoffrey D. Price

Computational methods can now make detailed and accurate predictions of the structures of inorganic materials. Electronic-structure calculations are being performed for increasingly large systems, and simulation methods based on 'effective potentials' may now be used to model complex materials. One of the most exciting fields of application is mineralogy, which poses a major challenge to the predictive capacity of contemporary computational techniques.

THE past 10 years have seen significant progress in our ability to undertake accurate and increasingly predictive calculations of the crystal structures and properties of solids and of the defects and impurities that commonly control important aspects of their behaviour. It is now recognized that modelling techniques have the potential to contribute to the design and improvement of materials with specific characteristics and that they may be used to predict the properties of solids under extreme conditions, a topical example being the behaviour of minerals under the very high pressures and temperatures of the Earth's lower mantle. The development of the field has also been assisted by the recent enormous expansion in computer power, which has enabled calculations to be performed on complex materials whose study by theoretical techniques would have been inconceivable even five years ago.

The materials studied most extensively by theoretical methods are metals¹, for which band-structure techniques have led to detailed knowledge of their electronic structure and some success in prediction of their structures. Here we will be more concerned with inorganic non-metallic crystals, with an emphasis on the modelling of structural, elastic, lattice dynamical and thermodynamic properties. We shall summarize the main techniques employed and illustrate their use by several recent examples, giving particular emphasis to the field of mineralogy, which poses some of the most exciting challenges to the current methodologies. Our aim is to answer the following questions. How well can existing techniques model the structure and properties of inorganic solids? What are the main problems and limitations? What future areas of application can be envisaged?

One can distinguish two general classes of techniques: electronic-structure calculations, which attempt, at some level of approximation, to solve the Schrödinger equation for the system; and simulations, which make use of 'effective potentials' that describe the interatomic forces in the system and which may be used to predict structural and dynamical properties of the solid using essentially classical techniques. Interactions between the two approaches are increasingly strong and indeed many of the most important future areas of application will require concerted use of both types of method. We shall describe both approaches, but with a degree of emphasis on simulations.

Electronic-structure calculations

Two approaches may be adopted in attempting to solve the Schrödinger equation in solids. In the first, a cluster of atoms representing the solid is studied; in the second, periodic boundary conditions are applied to the primitive unit cell (or to a supercell). Both approaches are used widely in contemporary studies.

Cluster calculations. These methods, which have been in use for over 20 years², are simple in concept. Once a suitable cluster has been defined, standard quantum-chemistry techniques can be used to identify the geometry of minimum energy. By examining the cluster wavefunction, the electron density distribution

and other aspects of the bonding may be investigated; and by varying the geometry of the cluster, interatomic potentials and force constants may be deduced. Indeed, such calculations are being used increasingly to provide the potentials used in simulation studies.

For cluster calculations, there are three choices to be made:

- (1) Choice of cluster size. Obviously the larger the cluster, the more closely are the calculated bonding properties likely to resemble those of the real infinite crystal. There will generally be a trade-off, however, between the cluster size and the level of sophistication of the calculation³.
- (2) Choice of cluster termination. As the cluster is a fragment of the solid, the peripheral atoms either will have unfilled valence shells ('dangling bonds') or will be charged. It is usually desirable to saturate dangling bonds and if possible to 'embed' the cluster by representing in some way the effects of a surrounding lattice. To achieve the former, hydrogen atoms are commonly used, although this may be unnecessary for more ionic systems in which the peripheral atoms may simply be closed-shell ions. See refs 2, 3, 4, 6 and 8 for further details and discussion.
- (3) Choice of quantum-mechanical technique. Many recent cluster calculations on solids have used Hartree-Fock methods and computational techniques developed to describe the quantum chemistry of small molecules. These methods use a molecular wavefunction represented as a linear combination of atomic orbitals. Electron-electron interactions are calculated as Coulomb and exchange interactions, and a self-consistent wavefunction is obtained using an iterative procedure. The techniques range from semi-empirical procedures such as CNDO and MNDO, which ignore or parameterize many of the overlap integrals, to *ab initio* methods in which all integrals (above a threshold energy) are evaluated.

The principal shortcoming of the Hartree-Fock method is its neglect of the effects of electron correlation. These can be included by the use of multi-determinantal wavefunctions; but the computational cost is considerable. Valence-bond methods build correlation more directly into the wavefunction, and cluster calculations for solids using such techniques have been reported⁸. An attractive and increasingly popular approach is to use the local-density approximation (LDA) of density functional theory. This is based on the demonstration by Hohenberg and Kohn⁹ that the ground-state energy of a system is a unique function of its electron density (strictly, the energy expression is a functional, as the electron density is itself a function of position). A self-consistent density is calculated using the variational principle (that is, by minimizing the total energy). Electron exchange and correlation energies can be evaluated by inserting the local density at a given position into the expressions appropriate for a uniform electron gas at that density. It is difficult to assess exactly the magnitude of the error introduced by this local approximation; but the method does involve the correlation energy directly, and it is enjoying considerable success in modelling the ground-state properties of solids.

Moreover, the efficiency of the method in locating minimum-energy geometries has been greatly enhanced by the work of Car and Parrinello¹⁰, which borrows ideas from the molecular-dynamics simulation technique. Cluster calculations have been reported that use LDA methods¹¹, but most applications involve periodic systems (see below).

The question of the relative merits of LDA and Hartree-Fock methods is controversial. A good recent review is given by Jones and Gunnarsson¹². LDA methods unquestionably have the advantage of being computationally cheaper, and therefore permit calculations at the *ab initio* level on larger systems. Their inclusion of correlation effects directly is a further substantial advantage. For these reasons, LDA methods have been used more widely than Hartree-Fock methods in recent work on solids. The latter methods do, however, have a substantial part to play, as is clear from the recent studies of SiO₂ described below.

Periodic boundary calculations. Both Hartree-Fock and LDA methods may now be used routinely to calculate the energies of an infinitely and periodically repeated unit cell. The most notable work using periodic-boundary Hartree-Fock calculations is that of Pisani and co-workers¹³, whose CRYSTAL code is now making major contributions to our understanding of bonding in solids; examples of its application to SiO₂ and silicates are given below. LDA methods have been applied extensively to semiconductors; below we discuss recent applications to crystalline silicon and silicate perovskites.

Simulations

In simulation methods, the detailed effects of electronic structure are subsumed into 'effective potentials'. The type of potential model used depends on the class of solid. Most of the properties of ionic solids may be modelled successfully by adding short-range pair potentials to the Coulombic forces acting between the ions, and by treating ionic polarization using the shell-model formalism developed by Dick and Overhauser⁷. For semi-ionic solids such as SiO₂ it is necessary to extend the treatment to include many-body terms, which may be treated by incorporating specific angle-dependent forces (depending, for example, on the O—Si—O bond angles). Alternative many-body formalisms include the 'triple-dipole' terms used originally by Axilrod and Teller¹⁴, which have been incorporated successfully into model potentials for the silver halides¹⁵. Approaches to modelling covalent solids are often based on valence-force potentials, which incorporate bond-stretching and bond-bending terms.

The most important step in any simulation is the parameterization of the potential. Empirical fitting procedures employing

model compounds have been widely used. There is, however, an increasing incentive to develop reliable methods for calculating directly the short-range component of the potential. The electron-gas methods of Wedepohl¹⁶ and Gordon and Kim¹⁷ have been used extensively and successfully by Mackrodt and coworkers¹⁸ to derive potentials for ionic solids. Quantum-mechanical cluster calculations are, however, being applied increasingly to the development of interatomic potentials; the strategy is to vary the geometry of the cluster in a systematic way and to fit the resulting potential-energy surface to an effective-potential model. There are many examples of such calculations, including the recent work of Lasaga and Gibbs¹⁹, who performed detailed studies on the Si(OH)₃—O—Si(OH)₃ molecule in order to derive potential parameters for SiO₂, the modelling of which will be discussed in greater detail below.

Considerable progress has been made in developing interatomic potentials for metals. Here, tight-binding calculations have been found to justify some of the empirical approaches, and there is growing use of 'embedded atom' models based on effective-medium concepts. Several discussions can be found in ref. 1, and Finnis *et al.*²⁰ have provided a good review.

Simulation codes are generally based on one of three techniques: energy minimization, molecular dynamics and Monte Carlo methods. Energy minimization simply involves generating the configuration of lowest potential energy for a given potential model. Molecular dynamics methods, which include kinetic energy explicitly in the simulation, are widely used to study fast diffusion processes in solids²¹ and properties of materials at high temperatures, for which the techniques that assume harmonic lattice vibrations are no longer valid. These methods follow molecular motions by solving Newton's equations of motion in discrete time steps. The Monte Carlo technique is essentially a method of computational statistical mechanics in which changes in the structure are successively made at random, with the configurations being accepted or rejected depending on an energy criterion. It is used most appropriately for disordered systems, recent examples being the study of sorption in porous solids²² and vacancy distribution in heavily doped fluoride oxides²³.

A particularly successful simulation technique for studies of the solid state is the Mott-Littleton⁵ method for studying defect properties. The approach combines energy minimization of a localized region of the lattice around the defect with a continuum treatment of the polarization of the remainder of the crystal. Reference 24 gives a comprehensive review.

Simulation methods are in general computationally cheaper than electronic-structure techniques. Moreover, they can yield information on time-dependent and thermodynamic properties

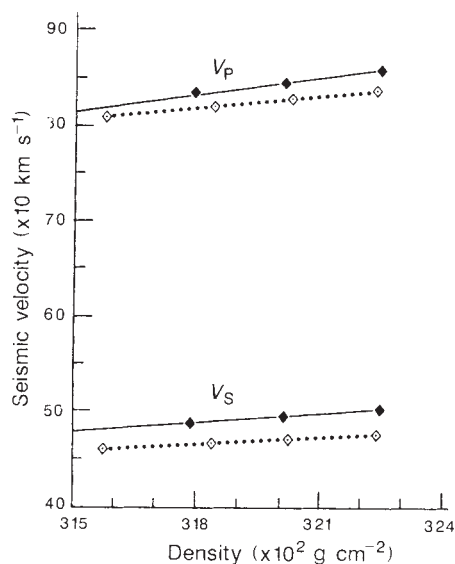


FIG. 2 Predicted (dotted line) and observed (solid line) P- and S-wave velocities of forsterite as a function of density⁶⁰.

which are inaccessible at present from direct quantum-mechanical formulations. For the foreseeable future, they will be the natural method for investigating large and complex systems. And they will always be the more appropriate method for investigating those problems for which the essential physics can be described by an effective-potential model.

We shall now describe some recent applications of many of these techniques to problems of topical interest.

Electronic-structure studies of silicon

The ability of *ab initio* electronic-structure techniques to make detailed and accurate predictions of the properties of crystalline solids is shown most clearly in studies of silicon. Yin and Cohen²⁵ used *ab initio* LDA techniques to show that the diamond structure has the lowest energy, as found experimentally, and that the calculated lattice constant and compressibility are very close to (within 1% of) the experimental values. They also studied the variation of stability with pressure, demonstrating that the metallic, white-tin structure becomes stable at 100 kbar and that a hexagonally close-packed phase forms at 400 kbar. The latter prediction has been verified experimentally.

This work and that of others shows clearly that it is possible to predict, from first principles and without the use of parameterized models, the structures and properties of structurally simple materials containing light atoms. The use of pseudopotentials (which represent the effects of core orbitals in a simple and economical way) should extend the scope of such calculations to more complex materials containing heavier atoms²⁶.

Simulation of SiO₂

Most modelling studies of SiO₂ have been based on simulations using effective potentials, although a valuable set of Hartree-Fock calculations using the CRYSTAL code with periodic boundary conditions have been reported by Dovesi *et al.*²⁷. That study reproduced the structure of the material well; greater precision has been achieved in a recent study by Nada and coworkers (to be published). LDA methods (employing the Car-Parinello approach) have been equally impressive in modelling the structures and properties of cristobalite²⁸. Several simulations of SiO₂ have been reported recently, with goals ranging from a simple description of the equilibrium structures of the SiO₂ polymorphs²⁹⁻³¹, through a description of the equation of state of quartz and its polymorphs³²⁻³⁴, to studies of the behaviour of amorphous silica^{35,36} and even the rheological properties of quartz³⁷.

The polymorphs of SiO₂ pose a major challenge to modellers, because their bonding is neither classically ionic nor ideally covalent. Hence, simple pairwise-additive ionic models are unable to describe the structural and elastic features of these phases, whereas models that describe short-range interactions and the directionality of the Si—O bond but that ignore the longer-range components fail in their description of the lattice dynamics and thermodynamic properties. This has led to the development of empirical models that incorporate both ionic features and covalent or short-range, angle-dependent terms²⁹⁻³¹. Arguably the first, and possibly still the most versatile potential to describe satisfactorily the structural and thermodynamic properties of SiO₂ phases was developed in 1984 by Sanders and coworkers²⁹. In this potential, Si and O are assigned full, formal-valence charges, but the directionality of the Si—O bond is modelled by introducing a bond-bending term. The polarizability of the oxide ion is modelled by a shell-model potential. The model reproduces not only the structural and elastic properties of quartz³⁶ and other framework silica phases³⁸ but also their spectroscopic and thermodynamic properties. In addition, the potential can be used to describe the behaviour of the more complex magnesium silicates³⁹⁻⁴¹.

Other potential models, both empirical³² and based on quantum mechanics^{19,31}, have also been developed; but in general they have not been critically assessed against the model of

Sanders *et al.*, so it is difficult to determine whether they actually represent improvements. Thus, for example, Gibbs and Lasaga^{19,31} have performed quantum-mechanical simulations on 'silicate molecules' that can be considered as fragments of silicate minerals. Their calculations involve a Hartree-Fock energy-level solution of the Schrödinger equation, and provide a potential-energy surface that describes the interatomic interactions within the fragment. These calculations are successful in modelling the known behaviour of true silicate molecules (such as disilic acid, H₆Si₂O₇), and the resulting potential-energy surfaces have been fitted both by a simple ionic model and by an essentially covalent model. In common with previous studies, these workers find that a two-body ionic model is unsatisfactory and conclude that an angle-dependent, covalent model is preferable.

The importance of many-body interactions in determining the behaviour of silica phases has also been highlighted by the use of modified-electron-gas (MEG) approaches. A problem of the MEG approach is that the O²⁻ ion is not stable in isolation, and must be stabilized by being contained within a charged sphere (the so-called Watson sphere) which simulates the stabilizing effect of the 'Madelung potential', the potential experienced by the oxygen ion in a crystal lattice. Cohen³⁴ used the potential-induced breathing (PIB) modification of the MEG approach to model stishovite, a high-pressure phase of silica. In this model the oxygen interatomic potentials are parameterized in terms of the Madelung site potential: a change in the site potential induces a corresponding change in radius, or a 'breathing', of the Watson sphere. The PIB model potentials include not only pairwise-additive interactions but also many-body effects, and as a result it successfully simulates trends in the elastic constants of alkaline earth oxides, which cannot be modelled by simple two-body potentials⁴². Jackson and Gordon³³ have used a double-shell modification of the MEG method to describe the directionality of the Si—O bond, and have thus simulated the equation of state of quartz and coesite (another high-pressure phase).

From both the empirical and non-empirical methods that have been reported to date, it is obvious that a successful model of silica needs to describe both the ionic nature of the material (to model the defect, vibrational and thermodynamic properties) and the directionality of the bonding. It is unfortunate that no critical comparison has been attempted of the merits and shortcomings of the many potential models that now exist. But a more general study of potential modelling led Stoneham and Harding⁴³ to conclude that "at present the best empirical potentials have a far greater demonstrated accuracy than explicit solutions to the Schrödinger equation". This point seems to be echoed by Lasaga and Gibbs¹⁹, who point to the close agreement between their quantum-mechanically derived Si—O potential and the earlier empirical potential of Parker⁴⁴; they conclude that

TABLE 1 Properties of MgSiO₃ perovskite

	Calculated ⁴⁶ (GPa)	Experiment ⁴⁷ (GPa)
C_{11} *	531	520
C_{12}	44	114
C_{22}	531	510
C_{13}	143	118
C_{23}	166	139
C_{33}	425	437
C_{44}	237	181
C_{55}	249	202
C_{66}	136	176
K †	249	245
μ ‡	192	184

* C_{ij} , elastic constants.

† K , bulk modulus.

‡ μ , shear modulus.

the "agreement supports the use of quantum-mechanical calculations as a framework within which to shape interatomic potentials".

Quantum-mechanical calculations on Mg silicates

Much recent research within the Earth sciences has aimed to determine the fundamental processes and mechanisms involved in mantle dynamics, lithospheric motion and plate tectonics. Significant advances have been made recently in our understanding of mantle phases, but future progress in resolving important geophysical problems depends upon the ability to obtain highly accurate values for the seismic and defect properties of minerals under pressure and temperature conditions appropriate to the mantle. To reproduce this environment experimentally represents a considerable challenge; as the prospect of overcoming many of the problems remains remote, the development of theoretical and computer-based atomistic models of high-density mantle-forming silicates has been given much attention. These studies should provide a more accessible way of establishing the properties of silicates at extremes of pressure and temperature, and should elucidate the microscopic or atomistic characteristics of mantle materials, which determine their bulk macroscopic and thermodynamic behaviour.

The work of Nada (to be published) and of Cohen *et al.*⁴⁵ exemplifies the current state of *ab initio* total-energy calculations on crystalline magnesium silicates. Both authors have investigated the electronic structure of high-pressure polymorphs of MgSiO_3 ; these are of particular interest as they contain octahedrally coordinated Si (Fig. 1). Nada's study of the ilmenite phase uses periodic-boundary-condition Hartree-Fock techniques. The calculations give an energy-minimized unit-cell volume and a c/a axis ratio that are within 2% of those measured experimentally. The results also indicate that octahedrally coordinated Si is significantly more ionic than tetrahedrally coordinated Si. Cohen *et al.*⁴⁵ perform total-energy calculations for cubic magnesium silicate perovskite, using the linear augmented plane-wave (LAPW) method to describe the charge density and potential surface within the unit cell. Although this approach is extremely accurate, it has the disadvantage that it is computationally very intensive—the calculation of the energy of the cubic perovskite cell (containing only five atoms) takes 10–20 c.p.u. hours on a Cray-XMP supercomputer. Although it is useful in estimating the compressibility of the hypothetical cubic magnesium silicate perovskite and in revealing the predominantly ionic nature of the bonding in such highly coordinated silicates, this computationally intensive approach is not practical at present for the general study of geophysically important phases.

Simulation of magnesium silicates

Early model simulations of silicates concentrated on reproducing the structure and elastic properties of known minerals. This

requires a correct description of both the first and second derivatives of the potential-energy surface within the crystal. It was generally found, however, that either the structure could be reproduced at the expense of predicting elastic constants that were unrealistically stiff, or that reasonable elastic constants could be obtained at the expense of predicting a structure with an unrealistically low density. With the realization of the need to account for many-body interactions, however, it has become possible to obtain models that predict zero-pressure, zero-temperature densities to within 2%, and elastic constants to within 5–10%, of those determined experimentally. The ability of computer models to predict the physical properties of mantle-forming phases was illustrated recently by a PIB model calculation of the elastic properties of magnesium silicate perovskite⁴⁶. Subsequent experimental data⁴⁷ (Table 1) are in very close agreement with the values calculated.

Excellent potential models have been developed by several workers^{38–40,48–56} and have been applied to most important mantle phases. It is widely recognized^{57–59}, however, that if outstanding geophysical problems, such as the nature of chemical layering in the mantle, are to be resolved, very accurate constraints must be placed on the thermal-expansion coefficients and elastic constants of mantle phases. For example, a 10% uncertainty in dK/dP for magnesium silicate perovskite (K being bulk modulus and P pressure), or an uncertainty of $\pm 2.0 \times 10^{-5}$ in its thermal-expansion coefficient, has an effect on the calculated seismic behaviour equivalent to a change in the chemical composition of the lower mantle from pyroclitic to chondritic. Thus, despite being able to predict equations of state and seismic velocities with unprecedented accuracy (Fig. 2), modellers find themselves in the frustrating position of still being unable to use their calculated equations of state to discriminate between various compositional models of the mantle.

In contrast to these limitations, computer simulations are making an immediate contribution to the prediction of phase relations and of defect properties, where experimental results are poorly constrained. The experimental phase diagrams of the $(\text{Mg,Fe})\text{SiO}_3$ and $(\text{Mg,Fe})_2\text{SiO}_4$ systems, for example, are still subject to considerable uncertainty because of the difficulty in producing consistent pressure and temperature calibrations. The success of Price and co-workers^{39,40} in reproducing the heat capacities of minerals using potentials based on the Si–O potential of Sanders *et al.*²⁹ means that related thermodynamic properties, such as entropy, are also predicted accurately. By accurately simulating cell volumes as well as free energies, these models can be used not only to predict the Clausius–Clapeyron slopes of phase boundaries (that is, dS/dV), but also the complete phase diagram (Fig. 3).

As the viscosity and rheological behaviour of the mantle is likely to be determined by diffusion, which is controlled in turn by the intrinsic disorder within mantle minerals, calculation of the formation and migration energies of these intrinsic defects

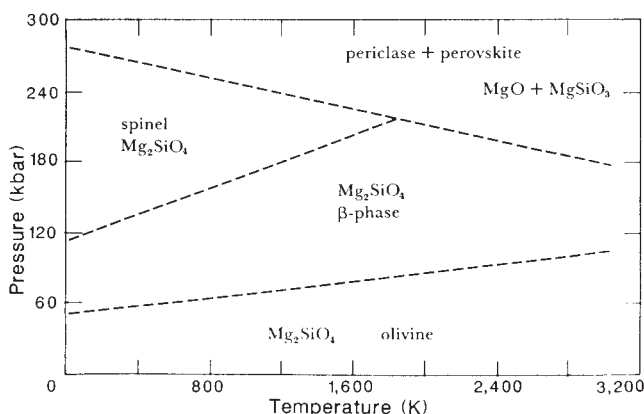


FIG. 3 The phase diagram for the magnesium orthosilicate system, calculated using free-energy simulation methods employing lattice-dynamical techniques.

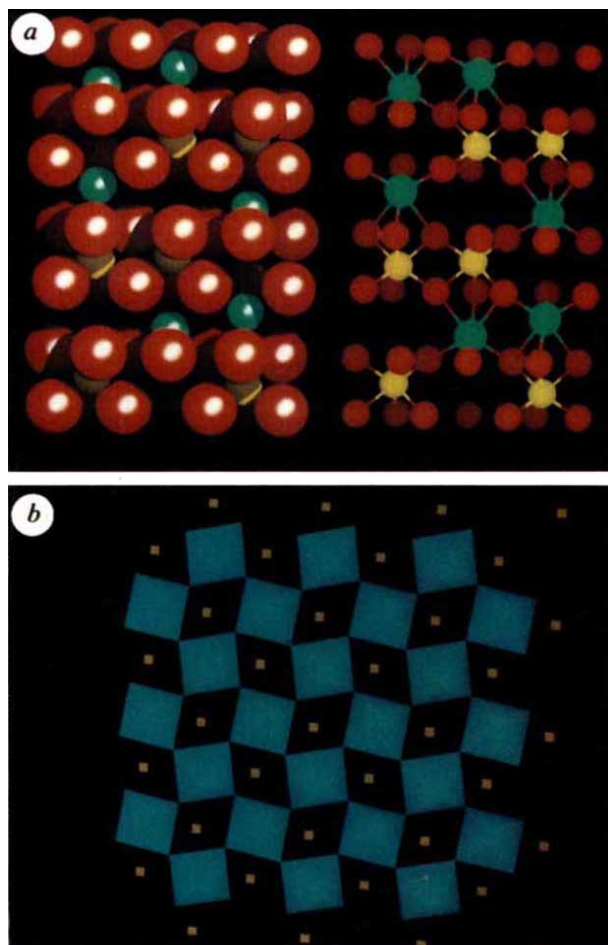


FIG. 1 The experimentally determined structures of *a*, the ilmenite phase and *b*, the perovskite phase of MgSiO_3 . In *a* the yellow spheres are Si, the green spheres are Mg and the red spheres are O. The diagram on the left shows the relationship of the crystal structure to the close-packed oxygen sublattice. In *b* the blue polyhedra represent octahedrally coordinated Si and the yellow squares indicate Mg^{2+} ions.

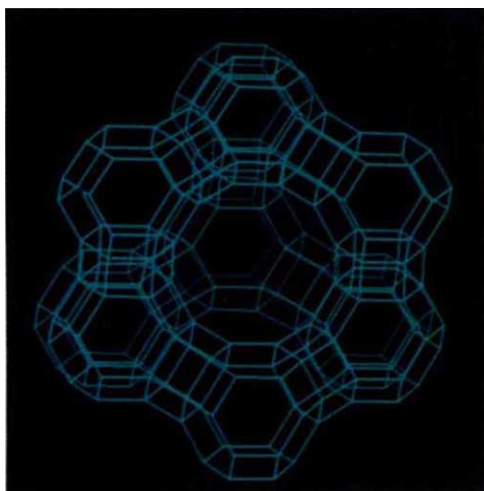


FIG. 4 The crystal structure of zeolite Y. This synthetic aluminosilicate is isostructural with the rare mineral faujasite.

is of considerable importance. The Mott-Littleton methodology has been used in association with the Si-O type potentials to model the defect behaviour of forsterite and magnesium silicate perovskite^{60,61}. Calculations⁶⁰ for forsterite indicate that Mg^{2+} Frenkel pairs of defects are most favourable energetically, although a significant proportion of MgO Schottky defects is also expected. These calculations established the diffusion pathway for Mg^{2+} vacancies and interstitials and predicted an activation energy of 3.85 eV, in agreement with experimental studies⁶² of Mg diffusion in olivine (which yield activation energies ranging between 3.7 and 4.5 eV). Similarly, the predicted⁶¹ activation energy for O diffusion in magnesium silicate perovskite (0.8 eV) is almost identical to that found for O diffusion in the structurally related high-temperature superconducting oxides⁶³.

The movement of oxygen in the magnesium silicate perovskite lattice has also been studied recently by molecular dynamics^{64,65}. The object of these studies was to investigate whether magnesium silicate perovskite is a 'superionic' conductor (a solid material showing electrical conductivity of the same magnitude as a molten salt) at mantle pressures and temperatures. Although differing in detail, the general conclusion of the two studies was that, at typical lower-mantle temperatures and pressures, magnesium silicate perovskite is indeed a superionic conductor with a completely mobile oxygen sublattice. It is predicted that the conductivity of magnesium silicate perovskite in the lower mantle will be $\sim 100 \text{ S m}^{-1}$, in agreement with the lower-mantle conductivity inferred from the modelling of temporal variations of the geomagnetic field⁶⁶.

Remarkable progress has been made in the past five years in the development of computer simulations of magnesium silicates. But to resolve the questions concerning the detailed composition of the mantle, it will be necessary to improve both the calculation and the description of interatomic potential-energy surfaces within minerals, and to develop a better treatment of anharmonic effects in the calculation of high-temperature properties. Simulations of defects and kinetic processes are, however, already able to add to our understanding of mantle behaviour. It will hopefully be possible in the future to investigate the role of iron in determining the nature of silicate perovskites, to investigate the onset of melting of mantle phases, to

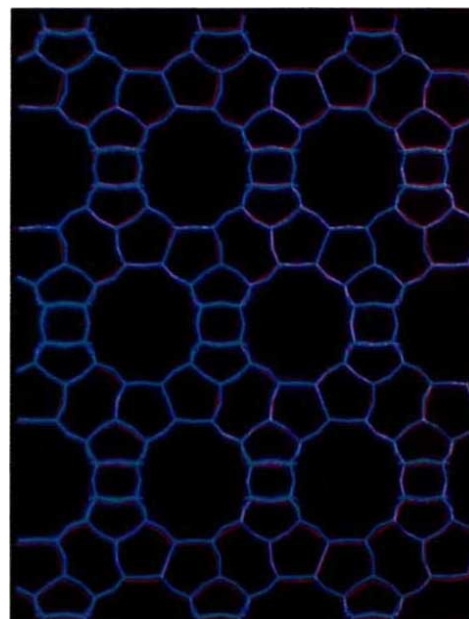


FIG. 5 The structure of 'polymorph A' of zeolite beta. The structure proposed by Newsam *et al.*⁷¹ is shown in blue; the energy-minimized structure⁷² is shown in red.

model silicate surfaces, and to extend the study of the effect of pressure on diffusion processes in silicates.

Modelling of zeolites

Zeolites are aluminosilicates (although an increasing number of almost purely siliceous materials are being prepared) with highly porous, 'framework' structures. Their current importance arises largely from their role as heterogeneous catalysts in the petrochemical industry. One of the most important examples is zeolite Y (Fig. 4), which is used extensively as a hydrocarbon-cracking catalyst. Modelling studies of these materials have concentrated on three aspects: the prediction of structures and stabilities of zeolite frameworks and of the positions of extra-framework cations; the modelling of the location and dynamics of sorbed molecules; and quantum-mechanical studies of the reaction pathways of sorbed reactive species. Here we concentrate on the first area; illustrations of the latter two can be found in refs 22 and 67.

Work over the past five years has shown that the three-body potentials derived for α -SiO₂ by Sanders *et al.*²⁹, when coupled with earlier empirical potentials derived for α -Al₂O₃, are able to yield excellent energy-minimized structural models for a wide range of zeolites. Structures studied include zeolite A, faujasite, silicalite and mordenite; a detailed discussion is given by Jackson and Catlow⁶⁸. Sanders and Catlow⁶⁹ have shown that simple energy-minimization procedures can also be used to model the location of extra-framework cations in zeolite X, and Jackson *et al.*⁷⁰ have modelled the location of Ni²⁺ in Ni-mordenite and its dependence on the Al distribution in the framework. Possibly the most exciting recent application has concerned zeolite β —a

structure that has long been controversial. Newsam *et al.*⁷¹ have shown that the structure is essentially an intergrowth of two polymorphs, both of which have been modelled successfully using energy-minimization techniques. The results of these calculations⁷² strongly support the structural models proposed by Newsam and Treacy⁷¹; the energy-minimized structure of the first polymorph (polymorph A), for example, is very close to that proposed by Newsam *et al.*⁷¹ (Fig. 5).

Conclusion

Electronic-structure and simulation techniques are now able to undertake realistic calculations on an increasingly wide and complex range of materials. The field is developing rapidly and ambitious projects such as full electronic-structure calculations on zeolites are becoming increasingly feasible. The most exciting opportunities will involve blending simulations with electronic-structure techniques to study, for example, defects in semiconductors and the reactions of molecules in zeolites. Accurate and predictive calculations of such properties can be envisaged in the near future.

Note added in proof: A recent publication (B. W. H. van Beest, G. J. Kramer and R. A. van Santen, *Phys. Rev. Lett.* **60**, 1955 (1990)) has described a quantum mechanically derived potential for SiO₂ which successfully described the crystal properties of the material and which has been carefully compared with the model of Sanders *et al.*²⁹. □

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A second class of synthetase structure revealed by X-ray analysis of *Escherichia coli* seryl-tRNA synthetase at 2.5 Å

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The three-dimensional crystal structure of seryl-transfer RNA synthetase from *Escherichia coli*, refined at 2.5 Å resolution, is described. It has an N-terminal domain that forms an antiparallel α helical coiled-coil, stretching 60 Å out into the solvent and stabilized by interhelical hydrophobic interactions and an active-site α - β domain based around a seven-stranded antiparallel β sheet. Unlike the three other known synthetase structures, the enzyme contains no classical nucleotide-binding fold, and is the first representative of a second class of aminoacyl-tRNA synthetase structures.

THE aminoacyl-tRNA synthetases are a group of enzymes, one for each amino acid, which specifically catalyse the esterification of an amino acid with a hydroxyl on the 3'-terminal adenosine of its cognate tRNA(s) (reviewed in ref. 1). The reaction requires ATP and the presence of Mg^{2+} for the formation of the aminoacyl-adenylate as an active intermediate. These enzymes thus have a crucial role in maintaining the fidelity of protein synthesis by their high degree of discrimination against incorrect tRNA aminoacylation, since messenger RNA is translated at the ribosome without reference to the amino acid attached to the tRNA. The need for such enzymes to mediate the translation of the genetic code was first suggested by Crick in 1958 as part of his adaptor hypothesis of protein assembly².

Much work has been undertaken to understand the molecular basis of the specificity of the aminoacyl-tRNA synthetases and the catalytic mechanism¹. Up until 1989, only two synthetase structures had been determined by X-ray crystallography. These were the dimeric tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* (YRSBS) and an active monomeric tryptic fragment of *E. coli* methionyl-tRNA synthetase (MRSEC). The structure of YRSBS is known in the presence of tyrosine³ and of tyrosyl-adenylate⁴ and that of MRSEC is known unligated and in the presence of ATP^{5,40}. Despite very little sequence similarity, these two enzymes possess a structural feature in common, namely a dinucleotide-binding fold based around a five-stranded parallel β sheet, a structural motif also found in dehydrogenases and kinases⁶. Part of this structure, a 44-residue β - α - β motif is essentially identical between the two enzymes⁷ and also contains in the first β - α loop, one of the few sequence motifs recognized in several aminoacyl-tRNA synthetases^{8,9}, His-Ile-Gly-His (the so-called HIGH region), which has important interactions with the ATP. This limited structural similarity is preserved in the most recent structure published, that of glutaminyl-tRNA synthetase from *E. coli* (QRSEC) complexed with its cognate tRNA and ATP¹⁰. This monomeric synthetase also contains a dinucleotide fold, similar to that found in YRSBS, and which makes many of the important contacts to

the three substrates, including the ATP in the vicinity of the HIGH region.

Because of a common dinucleotide-binding domain in the three aminoacyl-tRNA synthetase structures so far determined to high resolution, it has been suggested that all synthetases may have evolved from an ancestral catalytic domain that contained this structural motif¹⁰. The structure of the *E. coli* seryl-tRNA synthetase (SRSEC) described here shows that the evolutionary situation is more complex. SRSEC contains no HIGH sequence motif in its primary structure and no dinucleotide-binding domain in its tertiary structure. Indeed it has no obvious structural homology at all with the other three aminoacyl-tRNA synthetases, as its principal domain, which contains the active site, is built around a seven-stranded antiparallel β sheet. We present sequence evidence that other synthetases may have structural similarities with SRSEC and show that there are at least two families of aminoacyl-tRNA synthetase structures that have almost certainly evolved from different ancestors.

SRSEC is a dimeric enzyme of 430 residues per subunit (relative molecular mass 48,414). It charges specifically the five isoaccepting tRNA^{Ser} encoded by *E. coli*¹¹ and the recently discovered tRNA^{Secys} (ref. 12). The existence of a variety of anticodons amongst the tRNAs^{Ser} is consistent with experiments demonstrating that recognition between seryl-tRNA synthetase and the tRNA^{Ser} does not involve the anticodon¹³, unlike many other synthetases¹⁴. Sequencing, over-expression and crystallization of the enzyme have been described previously^{15,16}. Here we describe the three-dimensional fold of the protein as determined by X-ray crystallography using the method of multiple isomorphous replacement (MIR).

Structure determination

Information about the high-resolution native and derivative data collections, phasing statistics and refinement are given in Table 1 and the legend to Fig. 1 (which shows representative density from the solvent flattened MIR map). Full details of the structure determination and refined structure will be published elsewhere.

The two models derived from the native and ATP+serine data (see Table 1) are essentially identical, with no evidence that ATP, serine or seryl-adenylate are actually bound to the enzyme in the case of the ATP+Ser data. But there is a small molecule, probably a detergent molecule, bound to the protein in both structures (see below).

The current atomic model refined at 2.5 Å resolution contains 410 out of 430 residues, there being three undefined loops (residues 223–229, 272–279 and 377–381). Temperature factors are generally high with an average over all protein atoms of 30.4 Å². The overall structure meets the following strong criteria of correctness: (1) crystallographic *R*-factor of 18.8% for reflections between 7 and 2.5 Å ($I > 2\sigma$); (2) good geometry (for example, root mean square (r.m.s.) deviation in bond lengths of 0.018 Å); (3) the main chain of the molecule can be rebuilt using fragments from the database of well refined structures to within an r.m.s. accuracy of 0.3 Å for all main-chain atomic

positions (using the program MAXSPROUT; L. Holm and C. Sander, *J. molec. Biol.*), except that no fragment fits well the peptide centred at Ala 394. This is a peculiar three-glycine peptide (Gly-Ser-Gly-Leu-Ala 394-Val-Gly) between β strand A6 and helix H12, with an unusual *cis*-peptide between Gly 392 and Leu 393; (4) all but 10 of the ϕ/ψ angles are within the allowed zones, nine are close to the allowed zones and the exceptional case (Trp 363, $\phi = 57^\circ$, $\psi = -140^\circ$) seems to fit well the electron density in an omit map and (5) program DSSP¹⁷ reports that there are on average 74 main-chain hydrogen bonds per 100 residues, which is above average for proteins in the Brookhaven protein databank (C. Sander, personal communication) and indicates a well stabilized protein structure. α positions will be deposited in the Brookhaven protein databank.

Description of the structure

Seryl-tRNA synthetase consists of two domains. The first 100 N-terminal residues form an all α domain containing a remarkable solvent exposed arm consisting of an antiparallel coiled-coil of α helices and about 60 Å long. The rest of the molecule forms a large globular α - β domain built around an eight-stranded β sheet (seven antiparallel strands and one short parallel strand at one edge, forming part of the dimer interface). Overall the protein contains about 40% α helix and 20% β sheet (in the globular domain alone, residues 101-430, the amounts are 26% and 24%, respectively). Figure 2a shows the α backbone of the monomer and Fig. 2b shows the dimer viewed along the two-fold axis. Figure 3 shows a schematic diagram of the folding topology with the main secondary structural features indicated. **The helical arm.** The helical arm is composed of two very long antiparallel helices, H3 of 10 turns (residues 27-64) and H4 of nine turns (residues 69-100) joined by a short segment of extended chain (residues 66-68). The arm makes an angle of about 55° to the twofold axis; in the dimer, the two arms are nearly perpendicular with a distance of ~ 110 Å between their tips (see Fig. 2b). The arm structure completely lacks aromatic residues and 40% of the residues are charged. Helix H3 has an overall positive charge (nine positive, five negative), whereas helix H4

has an overall negative charge (four positive, nine negative). Including the loop, there is an overall net charge of -2 . Whereas helix H3 takes part in important crystal contacts, the outer surface of helix H4 is exclusively in contact with solvent, and consequently its temperature factors are higher.

The two helices in SRSEC form the longest antiparallel pair so far reported in the literature and curve around each other with a slight left-handed twist. They do not form a regular coiled-coil as found in fibrous proteins such as tropomyosin, myosin and fibrinogen¹⁸, in which the individual helices have a left-handed supercoil and form a parallel pair with a left-handed twist of pitch about 150 Å. The individual SRSEC helices have a right-handed supercoil and form an antiparallel pair with a left-handed twist. This geometry cannot extend indefinitely, but can occur for a limited length (provided the supercoil pitch is large) while maintaining close interhelical contact. Indeed the (straight) axes of the major helices in the individual coiled-coils in SRSEC make an angle of 130° and the interaxis separation is 8.8 Å in the middle, diverging to about 10.5 Å at the ends.

Apart from the normal α -helical hydrogen bonds, the helical arm is stabilized by interactions of three types: (1) a core of hydrophobic interactions, (2) interhelical salt bridges and hydrogen bonds and (3) intrahelical salt bridges and hydrogen bonds. Interactions of type (2) and (3) will be described in more detail elsewhere, as well as the crystal contacts that the arm makes. Here we discuss only the main hydrophobic interactions that occur in the arm (Fig. 4a).

The legend to Fig. 4a shows the sequence of the two helices arranged in heptads as normally used in discussions of coiled-coils¹⁸. Helix H4 has heptads following rather closely the average composition in α fibrous proteins¹⁸ with exclusively hydrophobic residues in positions a and d. Helix H3 has a less characteristic sequence, in particular in position a, normally hydrophobic in a classical coiled-coil, there are two arginines (having long aliphatic chains) and a threonine. The interhelical hydrophobic interactions involve all residues in position a and d from both helices and result in a continuous hydrophobic

FIG. 1 Stereo view of the solvent-flattened MIR electron density at the beginning of β strand A7 including *cis*-proline 257, with the current atomic model superposed. This density was obtained, using the ATP + Ser F_{obs} , after repeated cycles of solvent flattening combined with gradual phase extension to 2.5 Å starting with the 2.8 Å MIR map. The contour level is 0.85 σ .

METHODS. After initial data reduction, all crystallographic calculations were performed with the CCP4 program package (Daresbury Laboratory). MIR phases to 2.8 Å were calculated using three heavy-atom derivatives, ethylmercury chloride (two sites), dysprosium chloride (one site) and uranyl-acetate (a compact cluster of nine sites) and the resultant electron density map was improved by solvent flattening³⁷. Anomalous differences were included for the dysprosium and uranyl derivatives. An initial model was built using the BONES and fragment fitting options of FRODO³⁸ and subsequently refined using the GROMOS molecular dynamics refinement package³⁹. The current *R*-factor is 18.6% for the native data (all data 6-2.8 Å, no solvent, individual temperature factors refined) and 18.8% for the ATP + Ser data (all data with $I > 2\sigma$ between 7-2.5 Å, individual *B*-factors refined, 95 solvent molecules, *R* = 20.2% for all measured data).

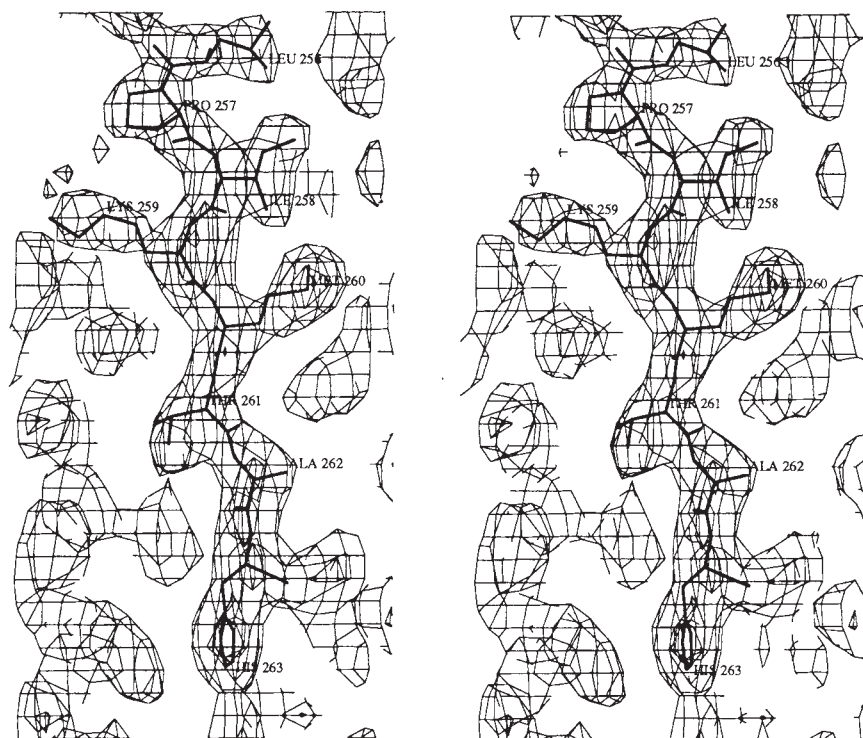


TABLE 1 Statistics of data collection and data analysis

Derivative	Native*	DyCl ₃ †	UO ₂ Ac‡	EtHgCl§	ATP + serine
Detector	Xenonics¶	Film *	Xenonics	Xenonics	Imageplate**
Wavelength (Å)	1.54	1.43	1.54	1.54	0.96
Total No. reflections/No. crystals	38,631/2	49,034/12	30,128/1	31,057/2	72,569/2
Unique reflections (% complete)	18,573 (88%)	17,918 (90%)	18,269 (91%)	13,953 (77%)	28,046 (98%)
Resolution limit (Å)	2.75	2.8	2.8	2.9	2.5
<i>R</i> _{merge}	0.059	0.059	0.068	0.067	0.059
Number of heavy atom sites		1	9	2	—
Isomorphous difference‡‡		0.11	0.20	0.11	0.11
<i>R</i> _{Cullis} §§		0.69	0.41	0.67	
Anomalous scattering used?		Yes	Yes	No	
Phasing power¶¶: all reflections to 2.8 Å (3.1–2.8 Å shell)		1.43 (1.09)	5.79 (4.06)	1.50 (0.94)	
Mean figure of merit for 16,054 reflections to 2.8 Å (3.1–2.8 Å shell)	0.69 (0.50)				

* SRSEC crystallizes in monoclinic space-group I2 (the body-centred equivalent of standard space-group C2) with unit cell parameters of $a=128.1$ Å, $b=90.7$ Å, $c=69.8$ Å, $\beta=91.1^\circ$. Crystals are routinely obtained by equilibration of a 1% protein solution in 67 mM Tris, 50 mM HCl, 10 mM MgCl₂, 1 mM DTE pH 7.6, 1 M ammonium sulphate and 1% of a particular batch of octyl-glucoside (practical grade, Pfanstiehl) against 52% saturated ammonium sulphate at room temperature. A 5 min centrifugation in a Beckman Airfuge at maximum speed is necessary before equilibration, to remove excess crystal nuclei. The detergent sample has been analysed by gas chromatography and is an impure mixture of ~40 components; the active ingredient has not been identified. After growth the crystals can be transferred to mother liquor, which does not contain detergent, whereupon the a cell axis increases to 129.7 Å and the crystals are less stable. These observations explain the variability in cell dimensions described previously¹⁶. In the work described here, detergent was always present. The asymmetric unit is the monomer, implying a solvent content of about 65%.

† Soaked for 4 days in 10 mM dysprosium chloride.

‡ Soaked for 3 days in 0.8 mM uranyl acetate at pH6.8.

§ Soaked for 4 h in 0.01 mM ethylmercury chloride.

|| Soaked for 2 days in 4 mM ATP + 4 mM L-serine.

¶ Xenonics data were collected with 0.2° frames (120 s) and arbitrary crystal orientation and integrated with the XENGEN package.

* Film data were collected on synchrotron line D41 at LURE using a wavelength of 1.43 Å to enhance the anomalous signal from the dysprosium (L_{II} edge is at 1.444 Å). Oscillation photographs (2°) were recorded with rotation about the unique twofold axis to obtain accurate measurements of Bijvoet differences with a crystal-film distance of 90 mm and exposure times of 11–16 min. Films (three per pack) were scanned with pixel size 50 µm using an Optronics rotating drum scanner and processed using the Imperial College package including profile fitting. Only full reflections were used.

** Image-plate data were collected at the EMBL Hamburg Outstation on beamline X11 using the EMBL camera-scanner system. Images were recorded with rotation about the crystallographic a^* -axis in two passes: first a high resolution pass with oscillation range 2°, crystal-plate distance 200 mm and exposure times of 7–14 s and then a low-resolution pass with oscillation range 3°, crystal-plate distance 288 mm and an attenuated beam. A second crystal was used to complete the blind region. Reflections were integrated with the Imperial College package without profile fitting (adapted for the image-plate by the EMBL Hamburg Outstation). Full and summed partial reflections were used.

†† $R_{\text{merge}} = \sum \sum |I_{\text{obs}}(h) - \langle I(h) \rangle| / \sum \sum I_{\text{obs}}(h)$, summed over all measured reflections.

‡‡ Isomorphous difference = $\sum |F_{\text{nat}} - F_{\text{deriv}}| / \sum F_{\text{nat}}$.

§§ $R_{\text{Cullis}} = \sum |FPH_{\text{calc}} - FPH_{\text{obs}}| / \sum |FPH_{\text{obs}} - FP|$, where FPH and FP are the derivative and native structure factor amplitudes and the sum is over centric reflections only.

¶¶ Phasing power = f_n/E where f_n is the r.m.s. heavy atom structure factor amplitude and E is the r.m.s. lack of closure error. Phasing statistics are as given by the final run of the CCP4 program PHARE before calculation of the first MIR electron density map. Undoubtedly the results are biased by the strong phasing power of the uranium derivative. Indeed, this derivative in itself would have been sufficient to solve the structure, although the other derivatives were necessary to place the cluster of uranium atoms.

core. However, the relative longitudinal stagger of the two helices, combined with the fact that in an α helix the sidechains tend to point back to the N terminus, mean that the most direct interactions are between residues in position a in one helix with position d in the second helix (Fig. 4b). Of particular interest are a number of interhelical leucine–(iso)leucine pairs. For instance, the heptad repeat of three leucines (Leu 33, Leu 40 and Leu 47) in position d in the first helix interact, respectively, with a similar repeat of an isoleucine and two leucines (Ile 96, Leu 89, Leu 82) in position a in the second helix. There are two other leucine–isoleucine interhelical pairs (Leu 30/Ile 99 and Ile 58/Leu 71), as well as two occurrences of interhelical leucine–arginine interactions. In these interacting pairs, the side chains point towards each other (Fig. 4b). To avoid unfavourable contacts in the middle, they are systematically turned slightly outwards in opposing directions and lie side by side (Fig. 4a). This arrangement of interhelical interacting side chains is most appropriately called a ‘leucine ladder’.

The globular domain. The globular domain is an α – β domain comprising residues 101–430 built around a seven-stranded antiparallel β sheet (sheet A in Fig. 3), with a short eighth parallel strand at the dimer interface (see below). There are three other short antiparallel β sheets (B, C and D in Fig. 3) in this domain as well as five short helices and two long α

helices. One of the long helices (H7, residues 167–186) is perpendicular to the twofold axis at the top of the molecule and interacts with the symmetry-related helix as part of the dimer interface. The second long helix (H10, residues 301–319) forms a right-handed cross-over connection between β strands A6 and A2 and packs on the outside (top) surface of the β sheet. The three poorly defined loops are in this domain (residues 223–229, 272–279 and 377–381). Out of 19 prolines in the enzyme, there are two *cis*-prolines (Pro 211 and Pro 257), both preceded by leucines, in accordance with the marked preference in this position for hydrophobic residues unbranched at C β (ref. 19). **Dimer interface.** The most important inter-monomer interactions occur in the long insertion of 132 residues between β -strands A1 and A7. The antiparallel helices H7 (residues 169–186) and H7* (residues 169*–186*, where * indicates the second monomer) interact across the twofold axis by means of complementary charged and polar side chains. The other principal features of the dimer interface involve two short inter-monomer antiparallel β sheets. The first is a continuation of the main eight-stranded β sheet with strands A9* and A10* being hydrogen bonded to a short stretch (residues 260–261) of strand A8 (Fig. 2b). The second is a symmetrical six-stranded antiparallel sheet (visible close to the twofold axis in Fig. 2b) comprising strands B1, B2 and B3, which interact with their

TABLE 2 Homology of seryl-, threonyl- and prolyl-tRNA synthetases in the active site

		←βA7→															←disordered→										←βA6→									
SRSEC	254	D	D	L	P	I	K	M	T	A	H	T	P	C	F	R	S	E	A	G	S	Y	G	R	D	T	R	G	L	I	R	M	H	Q	F	D
SRSSC	265	E	Q	L	P	I	H	Y	V	G	Y	S	S	C	F	R	R	E	A	G	S	H	G	K	E	A	W	G	V	F	R	V	H	A	F	E
PRSEC	126	K	Q	L	P	L	N	F	Y	Q	I	Q	T	K	F	R	D	E	V	R	P	R	F					G	V	M	R	S	R	E	F	L
TRSEC	349	R	D	L	P	L	R	M	A	E	F	G	S	C	H	R	N	E	P	S	G	S	L	H				G	L	M	R	V	R	G	F	T
TRSSCM	148	N	E	L	P	L	R	F	S	D	F	S	P	L	H	R	N	E	A	S	G	A	L	S				G	L	T	R	L	R	K	F	H
TRSSC	435	R	E	L	P	W	R	V	A	D	F	G	V	I	H	R	N	E	F	S	G	A	L	S				G	L	T	R	V	R	R	F	Q
TRSBS2	351	R	D	L	P	I	R	M	A	E	F	G	Q	V	H	R	H	E	Y	S	G	A	L	N				G	M	L	R	V	R	T	F	C
TRBSBV	353	R	E	L	P	I	R	I	A	E	L	G	T	M	H	R	Y	E	M	S	G	A	L	S				G	L	Q	R	V	R	G	M	T
			+	*	*	+	+	+			+		+	+	*		*		+									*	+		*	+	+		+	

Homology of the sequences of seryl-tRNA synthetases from *E. coli* (SRSEC) and *Saccharomyces cerevisiae* (SRSSC), prolyl-tRNA synthetase from *E. coli* (PRSEC²⁵) and threonyl-tRNA synthetases from *E. coli* (TRSEC), *S. cerevisiae* both cytoplasmic (TRSSC) and mitochondrial (TRSSCM), and two enzymes from *Bacillus subtilis* (TRSBS2 and TRBSBV)³⁶. * Conserved residues, shown in bold type; +, conservative changes. The secondary structure in SRSEC is indicated.

symmetry-related counterparts (B3 and B3* being antiparallel at the interface).

Putative active site. There are neither crystallographic nor biochemical data on the sites of binding of any of the substrates (ATP, serine, tRNA) to SRSEC. But examination of the structure reveals a putative active site in the globular domain. Whereas one side of the β sheet is overlaid by the long helix H10 (residues 301–319) forming a convex surface, the other side forms the floor to a deep depression, rimmed by loops and visible in Fig. 5. Of particular note is the loop formed by residues 269–285, part of which is not clearly visible in the electron density map (residues 272–279). This loop contains sequence motifs conserved among certain other synthetases, suggesting its functional importance (see discussion). We imagine it to form a flexible lid over the active site region, which may be stabilized on binding of substrates. There are several basic residues in the putative active site (for example, Arg 268, 283, 397 and Lys 289), reminiscent of MRSEC, where there are seven lysines and one arginine in the active site⁴⁰. The cluster of nine uranium atoms, bound on soaking the crystals in uranyl acetate, and the dysprosium atom are in the putative active site.

There is strong residual positive electron density in the region of the active site of SRSEC, which is present in native crystals in mother liquor containing detergent, native crystals transferred to mother liquor without detergent and in crystals soaked with ATP and ATP + serine. The density is in the form of an elongated 'tadpole' about 13 Å long and because of its universal presence it is probably a very tightly bound detergent molecule, arising from the crystallization medium (see legend to Table 1). The position of this molecule is under the loop 269–285 (in particular under the strand Gly 280-Leu-Ile-Arg), with its tail buried in a hydrophobic pocket and its head in a more hydrophilic environment. The detergent molecule may be of the octyl-glucoside type.

In the crystal, important intermolecular contacts occur in the region of the active site. The end of the antiparallel arm from a symmetry-related dimer is inserted into the putative active site ('foot in mouth'). Within the dimer itself, the acidic extremity of the first disordered loop from one monomer (residues Glu 224-Glu-Glu-Ala-Asp 228) probably crosses over into the active site region of the other monomer. From these observations we suggest that because of the tight binding of the detergent molecule and the crystal packing constraints, the active site no longer has the conformational flexibility to allow true substrate binding in the crystal. Co-crystallization of SRSEC with its small molecule substrates (ATP and serine), in the presence of detergent, again shows no indication of substrate binding; in the absence of detergent, no crystals are obtained. This suggests that crystals only occur as a result of the, presumably small, conformational or surface effects of the detergent binding, which then precludes further substrate binding. In solution, the presence of detergent does not interfere with enzyme activity in the tRNA charging reaction. A low-resolution neutron diffraction study is being undertaken to determine whether there are other

detergent molecules bound less specifically, using methods previously applied to the detergent-containing crystals of the photosynthetic reaction centre²⁰.

Discussion

The structure described above shows that SRSEC is the first example of an aminoacyl-tRNA synthetase that does not possess a dinucleotide-binding fold, thus contradicting a recent proposition that all synthetases had a common precursor containing this fold¹⁰. Whereas in the dinucleotide-binding fold the active site is formed by the loops at one edge of a parallel β sheet, in SRSEC the putative active site is formed on top of the central part of an antiparallel β sheet. The radically different nature of these active site domains may imply important differences in the mode of binding of the ATP, the 3' end of the tRNA and even the catalytic mechanism. Thus there are at least two distinct families of synthetases, which may have evolved from different ancestral enzymes. Further evidence for the partition of the aminoacyl-tRNA synthetases into families comes from the existence of certain conserved motifs in known synthetase primary sequences. One group (including the enzymes for arginine, glutamine, glutamic acid, isoleucine, leucine, methionine, tryptophan, tyrosine and valine)⁹ possess the HIGH motif, which is generally very close to the N terminus of the protein and which has been demonstrated in the three-dimensional structures of YRSBS, QRSEC and MRSEC to be associated with a conserved structural feature (part of the dinucleotide fold). They also all possess a Lys-Met-Ser-Lys-Ser (KMSKS) motif implicated in the binding of the 3' end of tRNA²¹. A second group (including the enzymes for lysine, aspartic acid, asparagine, phenylalanine, glycine, alanine and histidine) lacks the HIGH motif but contains a conserved Gly-Ile/Leu-Glu/Asp-Arg (GI/LE/DR) motif^{22–24}. A survey of the ~40 known aminoacyl-tRNA synthetase sequences confirms the partition of the synthetases into two groups on the basis of these conserved motifs (P. Argus *et al.*, unpublished results).

There is a region of homology between the known sequences of seryl-, threonyl- and prolyl-tRNA synthetases (Table 2). Using the SRSEC structure we can assign this region to β strand A7 and the partly disordered loop (268–285) connecting β strands A7 and A6 (see Table 2 and Fig. 5). There are strictly conserved residues at the beginning of strand A7 and in the connecting loop between strands A7 and A6, which seems to form a flap over the putative active site. Pro 257 in SRSEC is preceded by a *cis*-peptide and the strict conservation of the Leu-Pro in the two other synthetases suggests that this particular structural detail is also conserved. There is also a motif Phe/His-Arg-X-Glu (F/HRXE) which is found in certain synthetases of the second class²³ and a motif Gly-Leu-X-Arg (GLXR) where X is hydrophobic or nonpolar; both strictly conserved arginines are in the putative active site in SRSEC.

The idea that the aminoacyl-tRNA synthetases fall into two distinct classes has been further elaborated recently with the

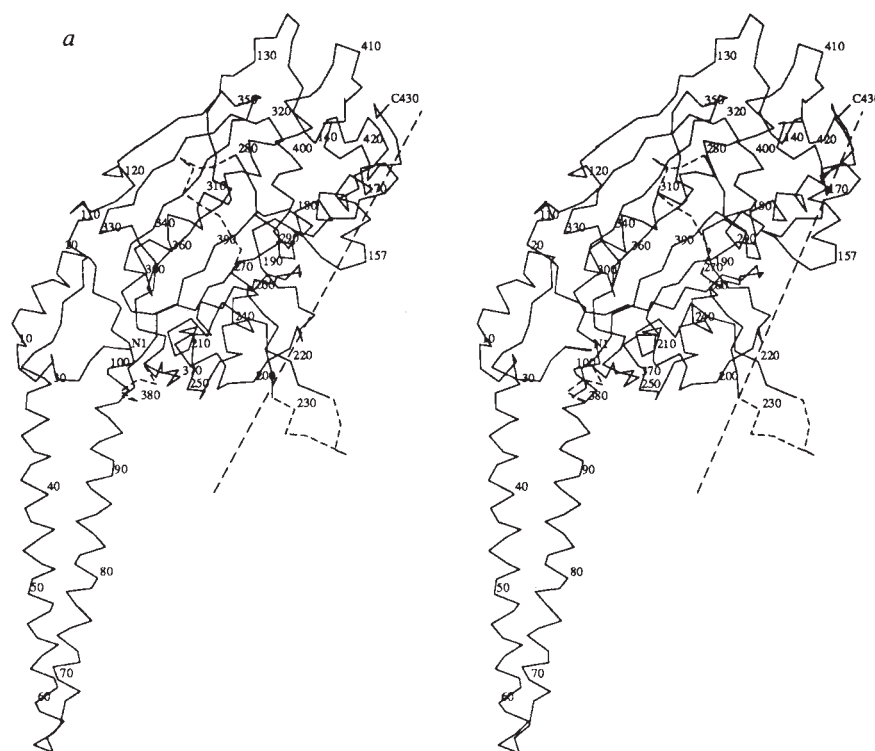


FIG. 2 *a*, Stereo view of $C\alpha$ backbone of one monomer of seryl-tRNA synthetase including the approximate position of the three ill-defined loops (dotted). The dimer twofold axis is shown (dashed). *b*, Stereo view of $C\alpha$ backbone of the dimer of seryl-tRNA synthetase viewed along the twofold axis. In one monomer the helical arm domain (mauve) and the globular domain (turquoise) are shown and in the other monomer the three chain breaks are shown (yellow). The main β sheet, with eight strands in one monomer and two strands in the other, is shown (red).

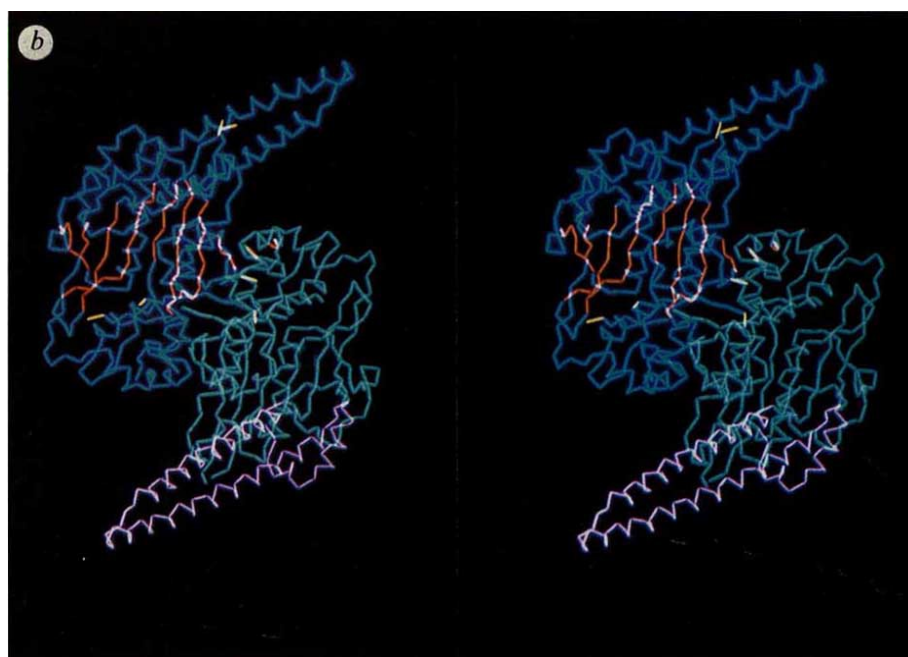
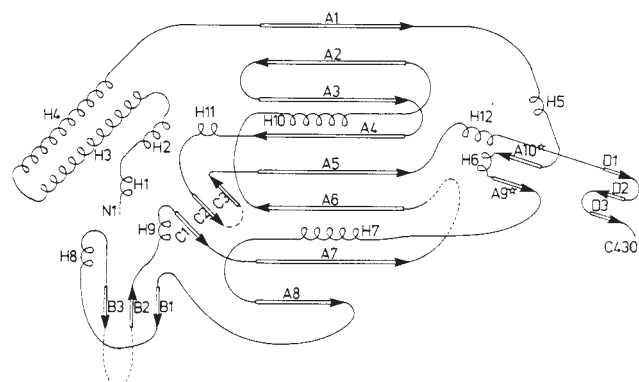


FIG. 3 Schematic diagram of main-chain fold of one monomer of seryl-tRNA synthetase. Principal secondary structural elements as determined by the algorithm of Kabsch and Sander¹⁷ are shown. Helices: H1 (residues 4–9), H2 (11–19), H3 (27–64), H4 (69–100), H5 (139–145), H6 (151–157), H7 (167–186), H8 (201–207), H9 (239–243), H10 (301–319), H11 (365–370), H12 (395–406). β strands: A1 (121–126), A2 (323–327), A3 (339–347), A4 (352–363), A5 (386–392), A6 (286–296), A7 (258–267), A8 (191–194), A9* (164–166), A10* (149–150), B1 (198–200), B2 (232–234), B3 (219–221), C1 (250–252), C2 (373–375), C3 (382–384), D1 (407–408), D2 (413–414), D3 (428–429). Relative directions of the β strands are indicated. Drawing by J. Sedita.



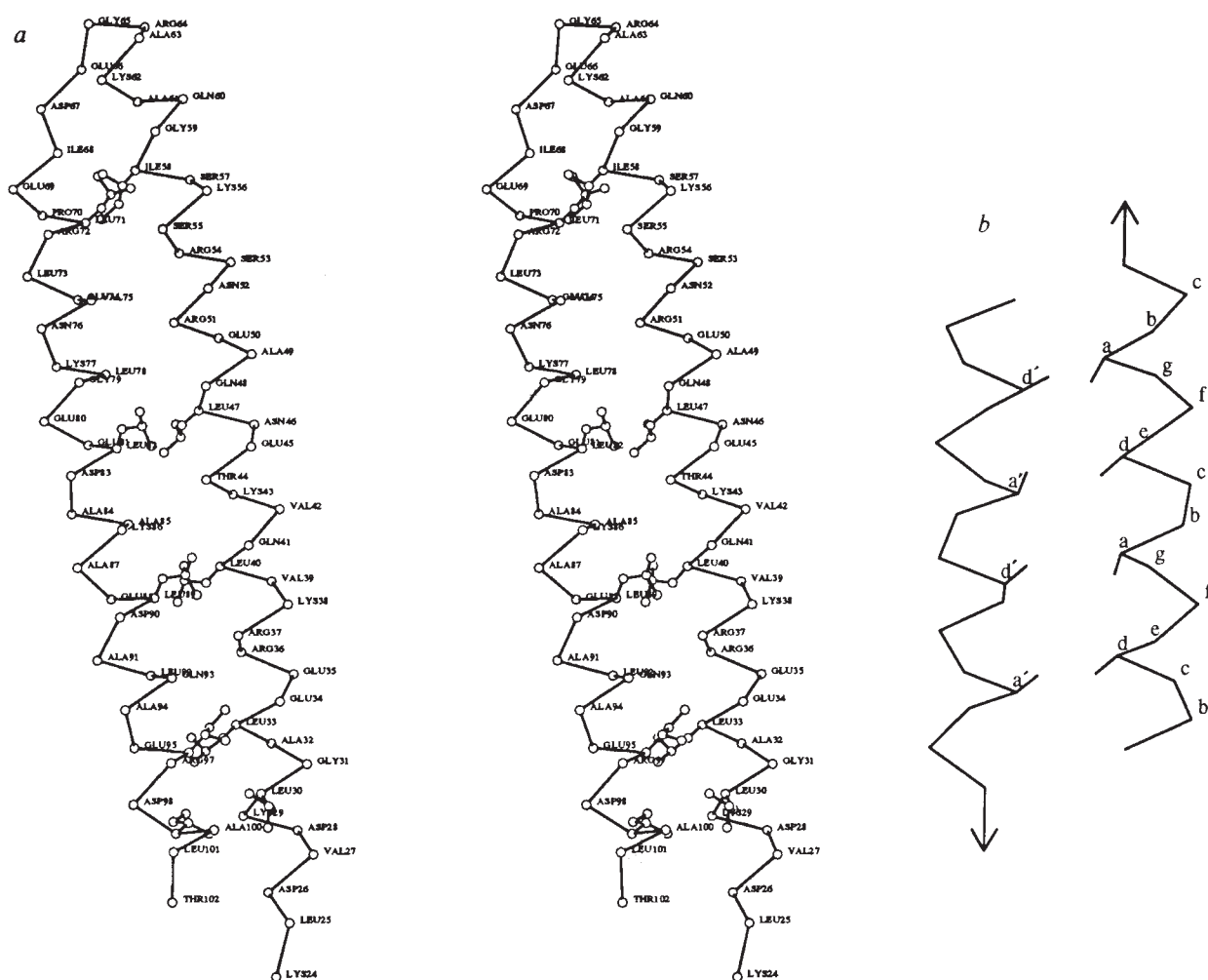


FIG. 4 *a*, Stereo figure of the C α positions in the helical arm showing interhelical leucine-(iso)leucine bridges. The sequence of the helices H3 and H4 arranged in heptads is as follows:

a	b	c	d	e	f	g
HELIX 3						
Leu 30	Gly	Ala	Leu 33	Val	Asp	Lys
Arg 37	Lys	Val	Leu 40	Glu	Glu	Arg
Thr 44	Glu	Asn	Leu 47	Gln	Val	Lys
Arg 51	Asn	Ser	Arg 54	Ser	Lys	Ser
Ile 58	Gly	Gln	Ala 61	Lys	Ala	Arg

HELIX 4

Glu	Pro	Leu 71	Arg	Leu	Glu
Val 75	Asn	Lys	Leu 78	Gly	Glu
Leu 82	Asp	Ala	Ala 85	Lys	Ala
Leu 89	Asp	Ala	Leu 92	Gln	Ala
Ile 96	Arg	Asp	Ile 99	Ala	

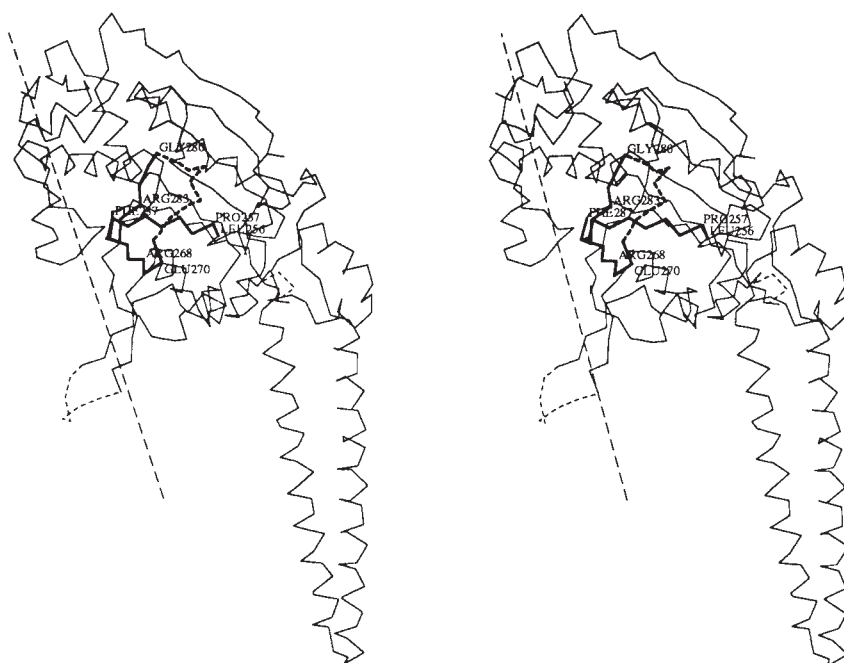
b, Schematic diagram showing the origin of the preferential interactions between heptad positions *a* and *d* in the first helix with, respectively, *d'* and *a'* in the second helix because of the particular longitudinal stagger of the antiparallel helices and the backward pointing direction of the C β atoms (see also *a*).

sequencing of the prolyl-tRNA synthetase from *E. coli* and the identification of three sequence motifs common to the second class of synthetases; the seryl-, prolyl- and threonyl- enzymes form a closely related subgroup within the second class²⁵. The degree of structural similarity between class 2 enzymes will soon become evident with the completion of the crystal structure determination of the aspartyl-tRNA synthetase: tRNA^{Asp} complex from yeast²⁶. The existence of different classes of synthetases has implications for the evolution of these enzymes and for the development of the genetic code. With a more limited database Wetzel²⁷ considered possible evolutionary relationships between the synthetases and noted the correlation of certain biochemical properties of synthetases (such as 2' or 3' ester formation and nature of the discriminator base) with codon family of the amino acid. The close sequence relationship of the enzymes for amino acids with XUX codons (notably

isoleucine, leucine, valine and methionine; all class 1 synthetases) has been established²⁸ and for some members of the XAX codon group²³. Three members of the XCX codon group (serine, threonine and proline) also form a closely related subgroup of class 2 synthetases (Table 2 and ref. 25).

The biological role of the helical arm of SRSEC is not yet experimentally demonstrated but the simplest hypothesis is that it is an extended strut to support the anticodon stem of the tRNA. In this role it would be analogous to the helical domain of MRSEC, which is composed of a bundle of more or less oriented helices⁴⁰. One important difference, however is that in MRSEC the only specific recognition between tRNA and synthetase is through the anticodon and the extremity of the helical domain⁴⁰, whereas in SRSEC the anticodon is unimportant for recognition¹³. This may help to explain the 'minimalist' nature of the SRSEC supporting arm. The helical arm is structurally

FIG. 5 Stereo view into the putative active site showing the region of homology between seryl-prolyl- and threonyl-tRNA synthetases (bold). The strictly conserved residues are marked (see Table 2). Disordered loops are dotted.



unusual and allows a detailed view of what is required to stabilize long helices in solution. This is of interest in relation to the class of DNA-binding proteins characterized by a sequence pattern comprising 3–5 leucine heptad repeats adjacent to a highly basic region^{29,30}. The repeated leucine motif (misnamed 'leucine zipper') has been demonstrated in some cases to mediate homo- or heterodimerization of domains through the formation of a parallel coiled-coil^{31,32}. Although SRSEC is not a member of this class of protein, the kind of interactions observed in the SRSEC arm may also be found in parallel coiled-coil structures with leucine heptad repeats.

Another RNA binding protein, ROP, involved in the regulation of replication of ColE1 plasmids, also contains a coiled-coil

of antiparallel helices twisted into a left-handed supercoil³³; the active form is a dimeric four-helix bundle. ROP binds weakly but specifically to the stem structure of the two RNA molecules involved in the regulatory mechanism and it has been proposed that simultaneous binding of the two RNA molecules to the ROP dimer mediates their hybridization³⁴. It is possible therefore that the antiparallel helical motif is found in other double-stranded RNA-binding proteins. Conversely, SRSEC itself may be involved in interactions with other RNA molecules. In this connection we note that threonyl-tRNA synthetase in *E. coli* negatively regulates its own translation by binding to an operator on its mRNA, part of which is structurally similar to the anticodon stem of tRNA^{Thr}, just upstream from the coding region³⁵.

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Molecular cloning of the microtubule-associated mechanochemical enzyme dynamin reveals homology with a new family of GTP-binding proteins

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A complementary DNA encoding the D100 polypeptide of rat brain dynamin—a force-producing, microtubule-activated nucleotide triphosphatase—has been cloned and sequenced. The predicted amino acid sequence includes a guanine nucleotide-binding domain that is homologous with those of a family of antiviral factors, inducible by interferon and known as Mx proteins, and with the product of the essential yeast vacuolar protein sorting gene *VPS1*. These relationships imply the existence of a new family of GTPases with physiological roles that may include microtubule-based motility and protein sorting.

THE cytoplasmic microtubules of mammalian brain tissue are composed of tubulin and a diverse set of microtubule-associated proteins (MAPs). Of these, three proteins are mechanochemical nucleotide triphosphatases: kinesin^{1,2}, dynein^{3,4} and dynamin⁵. Kinesin⁶ and dynein⁷ produce force in opposite directions along microtubules. They are therefore thought to be involved in anterograde and retrograde axonal transport, respectively, as well as other forms of intracellular movement (reviewed in ref. 8). Only the heavy chain of kinesin (relative molecular mass 110,000–135,000; M_r 110–135K), and several kinesin-like proteins, have been characterized at the level of primary structure^{9–14}. This has revealed the existence of a common force-producing 'head' domain of ~350 amino acids, although the location of this domain within the linear sequence of the polypeptide varies^{9–15}.

Dynamin has a microtubule-activated ATPase activity and binds microtubules in a nucleotide-dependent manner, like kinesin and dynein, although many of its properties are distinct from those of the other two proteins⁵. The principal polypeptide of dynamin is a species of M_r ~100K (here termed D100) which is a prominent component of mammalian brain microtubules prepared in the absence of added nucleotides. Preparations of D100 crosslink microtubules into regular bundles, which dissociate and elongate in an ATP-dependent manner⁵. The ATPase and sliding activities required an unidentified co-factor in addition to D100⁵.

To obtain further insight into the mechanism of action of dynamin, and to examine its potential relationship to other proteins of interest, we have obtained a cDNA clone encoding the entire D100 polypeptide. We report here the predicted amino-acid sequence of D100 and identify a novel family of D100 homologues.

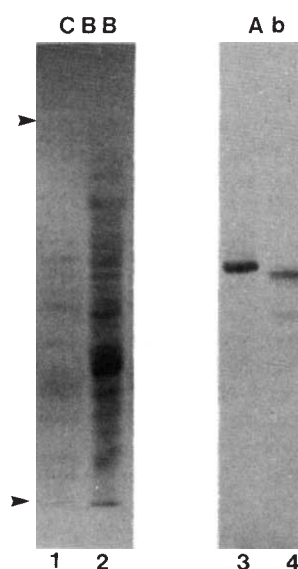


FIG. 1 Immunoblotting of dynamin-1 fusion protein and rat brain extract with affinity-purified anti-D100 antibody. Lanes 1, 3, Isopropyl thiogalactoside-induced plaques from clone λ ZAP-dynamin-1 (three plaques per lane). Lanes 2, 4, Adult rat brain extract (~5 μ g per lane). Proteins were stained with Coomassie brilliant blue (CBB, lanes 1, 2) or affinity-purified anti-D100 (Ab, lanes 3, 4). The top of the blot and the dye front are marked by arrowheads.

METHODS. Electrophoresis: A 9% gel⁴⁸ was immunoblotted⁴⁹ onto Immobilon PVDf (Millipore, Inc.) and stained with 0.1% CBB in 45% isopropanol, 10% acetic acid, or incubated with autoimmune rabbit antibody, affinity-purified⁵⁰ from an immunoblotted band of bovine brain D100⁵. Reaction products were visualized with alkaline-phosphatase-conjugated secondary antibody, as recommended by the manufacturer (Promega Biotec). Cloning: the rat brain cDNA expression library constructed in λ ZAP from size-selected (that is, >3 kilobase pairs) cDNAs was a gift from Dr K. Angelides. Roughly 3×10^5 recombinants were screened essentially as for λ gt11⁵⁰, but using *E. coli* strain BB4. Serum (see text) was used at a dilution of 1:500 or 1:1,000 at room temperature overnight. Fourteen antibody-positive clones (λ ZAP-dynamin-1–14) were plaque-purified to homogeneity. Both autoimmune and immune rabbit antibodies, which were affinity-purified using the fusion protein produced by λ ZAP-dynamin-1⁵⁰, reacted with D100.

Molecular cloning of D100

Two antisera were used in this study. The first was derived from an autoimmune serum identified while screening rabbits before immunization. Serum from one of seven animals stained D100 along with several other bands on immunoblots of calf and rat brain cytosolic extract. Antibody was used after

FIG. 2 Nucleic acid and deduced amino-acid sequence of the cDNA insert of clone λ ZAP-dynamin-1. The putative start (ACCATGG)¹⁶, stop (TGA), and polyadenylation (AATAAA) sequences are overlined. Amino-acid residues confirmed by direct sequencing of bovine D100 are underlined. METHODS. DNA sequencing: The recombinant pBluescript phagemid was excised from clone λ ZAP-dynamin-1 by the *in vivo* protocol of Short *et al.*⁵¹ and each strand sequenced with Sequenase (US Biochemicals) from a set of overlapping exonuclease III, *HincII*, *PstI*, and *PvuII* fragments; synthetic oligonucleotide primers were used to fill in gaps. Peptide sequencing: About 500 picomoles of the $M_r \sim 100$ K polypeptide of calf brain dynamin (prepared as in ref. 5) was further purified by preparative electrophoresis through a 7% sodium dodecyl sulphate polyacrylamide gel⁴⁸, then digested *in situ* in a second, 14% gel with *Staphylococcus aureus* V8 protease with an enzyme:substrate ratio of $\sim 1:400$ ⁵². This gel was electrobotted onto Immobilon-PVDF, which was then stained with Coomassie brilliant blue. A major peptide of $M_r \sim 30$ K was excised from the blot and sequenced on an Applied Biosystems Model 470A sequenator, to give the sequence: LAYM-NTNHEDFIGFANAQQRSSNQMNKKKASGNQ with an initial yield of 106 picomols. This bovine sequence differs from the derived rat sequence after Glu 482 (a likely V8 cleavage site) only by the replacement of an alanine for threonine 511.

	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350	360	370	380	390	400	410	420	430	440	450	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600	610	620	630	640	650	660	670	680	690	700	710	720	730	740	750	760	770	780	790	800	810	820	830	840	850	860	870	880	890	900	910	920	930	940	950	960	970	980	990	1000	1010	1020	1030	1040	1050	1060	1070	1080	1090	1100	1110	1120	1130	1140	1150	1160	1170	1180	1190	1200	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300	1310	1320	1330	1340	1350	1360	1370	1380	1390	1400	1410	1420	1430	1440	1450	1460	1470	1480	1490	1500	1510	1520	1530	1540	1550	1560	1570	1580	1590	1600	1610	1620	1630	1640	1650	1660	1670	1680	1690	1700	1710	1720	1730	1740	1750	1760	1770	1780	1790	1800	1810	1820	1830	1840	1850	1860	1870	1880	1890	1900	1910	1920	1930	1940	1950	1960	1970	1980	1990	2000	2010	2020	2030	2040	2050	2060	2070	2080	2090	2100	2110	2120	2130	2140	2150	2160	2170	2180	2190	2200	2210	2220	2230	2240	2250	2260	2270	2280	2290	2300	2310	2320	2330	2340	2350	2360	2370	2380	2390	2400	2410	2420	2430	2440	2450	2460	2470	2480	2490	2500	2510	2520	2530	2540	2550	2560	2570	2580	2590	2600	2610	2620	2630	2640	2650	2660	2670	2680	2690	2700	2710	2720	2730	2740	2750	2760	2770	2780	2790	2800	2810	2820	2830	2840	2850	2860	2870	2880	2890	2900	2910	2920	2930	2940	2950	2960	2970	2980	2990	3000	3010	3020	3030	3040	3050	3060	3070	3080	3090	3100	3110	3120	3130	3140	3150	3160	3170	3180	3190	3200	3210	3220	3230	3240	3250	3260	3270	3280	3290	3300	3310	3320	3330	3340	3350	3360	3370	3380	3390	3400	3410	3420	3430	3440	3450	3460	3470	3480	3490	3500	3510	3520	3530	3540	3550	3560	3570	3580	3590	3600	3610	3620	3630	3640	3650	3660	3670	3680	3690	3700	3710	3720	3730	3740	3750	3760	3770	3780	3790	3800	3810	3820	3830	3840	3850	3860	3870	3880	3890	3900	3910	3920	3930	3940	3950	3960	3970	3980	3990	4000	4010	4020	4030	4040	4050	4060	4070	4080	4090	4100	4110	4120	4130	4140	4150	4160	4170	4180	4190	4200	4210	4220	4230	4240	4250	4260	4270	4280	4290	4300	4310	4320	4330	4340	4350	4360	4370	4380	4390	4400	4410	4420	4430	4440	4450	4460	4470	4480	4490	4500	4510	4520	4530	4540	4550	4560	4570	4580	4590	4600	4610	4620	4630	4640	4650	4660
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FIG. 3 a, Alignment of the nucleotide-binding motif of D100 with those from GTP-binding proteins of several families. Dynamin-1, sequence derived from λ ZAP-dynamin-1 (see Fig. 2); Ras, human Ha-Ras p21 (taken from ref. 42); EF-Tu, *E. coli* elongation factor Tu⁵³; SRP54, the M, 54K subunit of the signal recognition complex^{20,21}; Gs alpha, the α subunit of the bovine brain Gs complex⁵⁴. **b**, Alignment of elements I and II of the D100 sequence with those of kinesin heavy chain⁹ and three kinesin heavy chain-like proteins¹¹⁻¹⁴. The kinesin heavy-chain family is found to contain GTP-binding element II, which is aligned with the comparable region of D100.

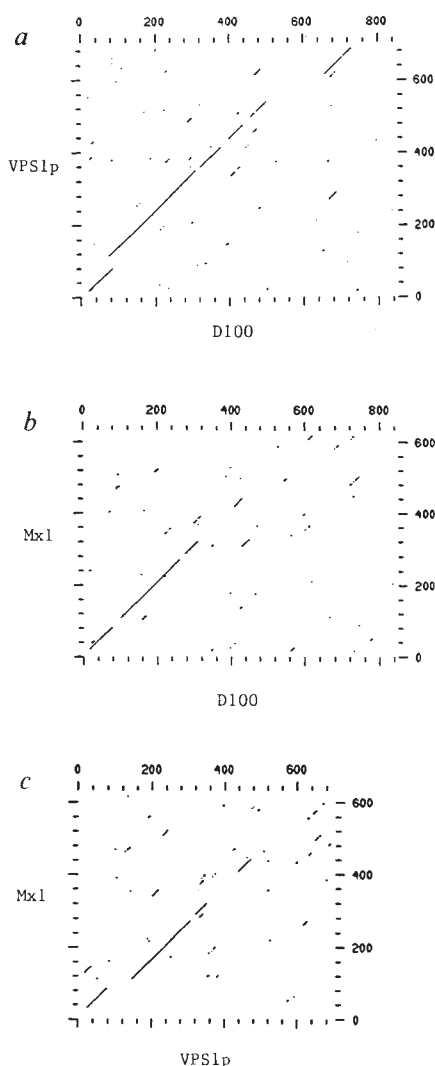
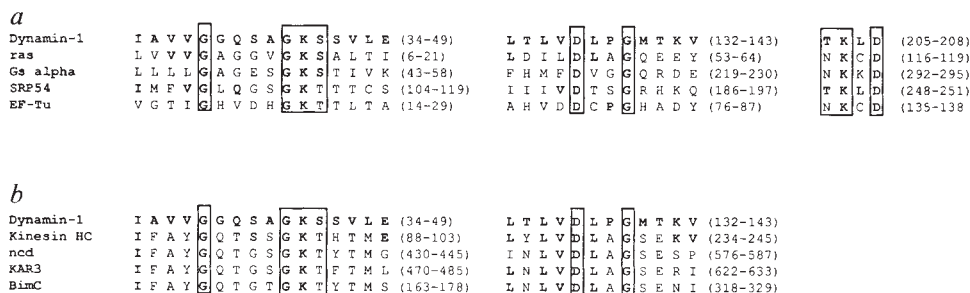


FIG. 4 Dot-plot comparisons of the predicted sequences of the rat D100 polypeptide, yeast VPS1p³³ and murine Mx1 (taken from ref. 27). **a**, D100 versus VPS1p; **b**, D100 versus Mx1; **c**, VPS1p versus Mx1.

METHODS. The following programs of the University of Wisconsin (Madison), Genetics Computer Group Sequence Analysis Software Package (version 6.1) were carried out on a VAX 11/750 computer²⁶: FASTA for database searches using the entire λ ZAP-dynamin-1 cDNA sequence or its derived polypeptide sequence; GAP for alignments; and DOTPLOT for dot-matrix comparison of pairs of sequences. Each of the DOTPLOT profiles was obtained with a window size of 32 and a stringency of 16.

affinity-purification from either electrophoretically purified D100 or bacterially expressed fusion protein (see below). A second rabbit polyclonal antibody was raised (C.A.C. and M. Stachniak, manuscript in preparation). Using this antibody, clones were selected from a rat brain cDNA expression library constructed in λ ZAP (see legend to Fig. 1). One of these clones was analysed in detail. It was judged to encode D100 by several criteria: (1) the β -galactosidase fusion protein produced by the clone was recognized by affinity-purified anti-D100 antibody preparations from both rabbits (see Fig. 1); (2) the clone contains an open reading frame beginning with a consensus eukaryotic translation initiation sequence (ACCATGG; ref. 16) that predicts an 851-amino-acid polypeptide of M_r 95,928, consistent with the size of the rat brain polypeptide observed by denaturing polyacrylamide gel electrophoresis⁵ (Fig. 2); and (3) the cDNA encodes an amino-acid sequence (residues 498-530) nearly identical to that obtained by amino-terminal microsequence analysis of a V8 proteolytic fragment of the bovine brain protein (see legend to Fig. 2). This cDNA clone, which contains a 3,215-base pair insert, was named λ ZAP-Dynamin-1.

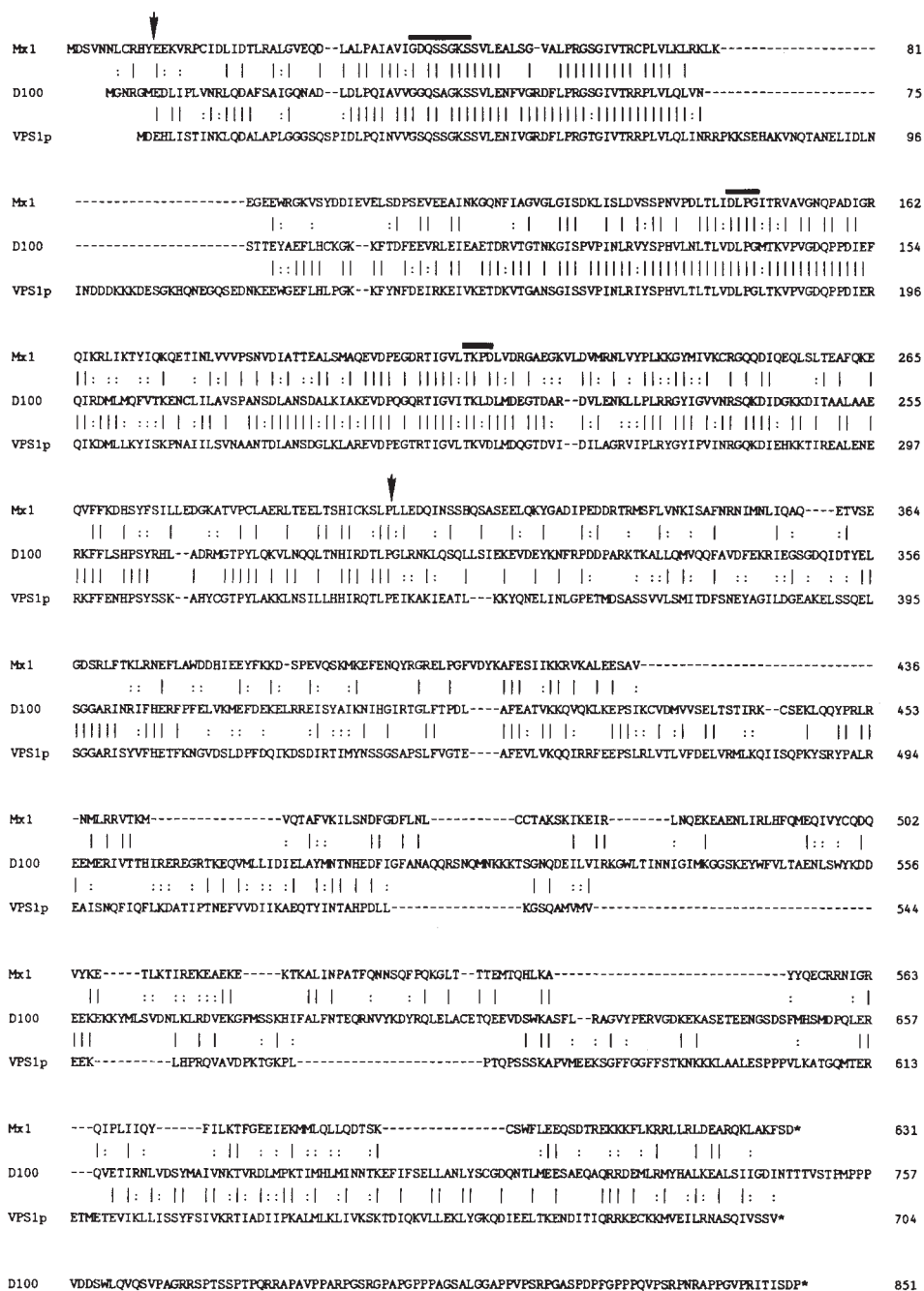
Relationship to other proteins

In view of the nucleotide-dependent interaction of D100 with microtubules, we inspected the predicted amino-acid sequence for features found in other nucleotide- or microtubule-binding proteins. Previous work indicated that dynamin is an ATPase⁵. The consensus sequence element indicative of ATP binding (the 'A-box' or 'element I')^{17,18} is GXXXXGKS(T) (single-letter amino-acid code, where X is any amino acid). This was found in the D100 sequence beginning at Gly 38. Surprisingly, two additional elements, DXXG (element II; beginning at Asp 136) and N(T)KXD (element III; beginning at Thr 205), which together with the first element are indicative of a GTP-binding motif¹⁹⁻²¹, are also present (see Fig. 3a).

Inspection of the D100 sequence revealed no overt similarity to the proposed microtubule-binding motifs of tau²², MAP 2 (ref. 23), MAP 1B heavy chain²⁴, or the trypanosome MAP p320 (ref. 25), all of which contain tandem repeats of lengths from 15 to 38 amino-acid residues. No repeated sequence elements were found in the nucleic acid or protein sequences reported here.

We found no extended similarity with kinesin heavy chain or any of the other members of the kinesin superfamily⁹⁻¹⁴. However, there is a sequence of 12 amino acids in D100, containing GTP-binding element II, that is quite similar to a region of the kinesin heavy-chain sequence near its proposed microtubule-binding site^{9,10} (Fig. 3b).

We searched the Genbank, EMBL, Swissprot and NBRF databases for D100-like sequences using the program FASTA²⁶. Two sequences with substantial similarity to D100 were found: the predicted products of the murine *Mx1* and *Mx2* genes^{27,28}.



glutathione-S-transferase-Mx1 fusion proteins (Fig. 6), consistent with the idea that the antibody represented a response to a previous viral infection. Mx1 is a minor histocompatibility antigen³⁵, but, to our knowledge, no other autoimmune reactivity with Mx proteins has been reported. We also note that stronger reactivity was observed with the D100 polypeptide than with the fusion proteins (not shown), suggesting that some of the determinants recognized by the antibody were modified in the brain polypeptide.

Functional considerations

The sequence analysis reported here indicates a nucleotide-binding site within D100, as expected from its mechanochemical properties⁵. This site is contained within a domain of ~300 amino acids also found in two other classes of polypeptides, VPS1p and Mx. The predicted sequence of the D100 polypeptide is unrelated to those of the rapidly growing family of kinesin-related proteins, with the exception of the small region we identify surrounding GTP-binding consensus element II (Fig. 3). No other similarity to the ~350-amino acid, force-producing head domain of kinesin, the common structural element in the kinesin family, was observed⁹⁻¹⁴. Although there is no direct evidence that the slightly smaller conserved domain in D100, Mx1 and VPS1p corresponds to a functionally analogous head, this is an appealing possibility.

A surprising result of the sequence analysis was the identification of GTP-binding consensus elements. Earlier work had revealed a microtubule-activated ATPase activity associated with dynamin⁵. GTPase activity was not evaluated, but there have been clear indications that GTP could also be used as a substrate. D100 could be extracted from microtubules with GTP as well as ATP, and GTP extraction is routinely used in D100 purification⁵. We have now found that purified D100 has a potent, microtubule-activated, GTPase activity (unpublished data). This result, along with other information, suggests that GTP is the likely physiological substrate for dynamin. In this context, we note that kinesin can also use GTP, although its affinity for ATP is higher, and therefore ATP seems to be its physiological substrate^{2,36}. Kinesin contains GTP-binding consensus element II (Fig. 3), though we find no evidence for element III. This indicates that use of GTP is possible without the third consensus element, which is known from crystallographic studies to be important to base specificity^{37,38}. In view of the fact that D100 and kinesin show limited similarity adjacent to element II, an additional role for this region, such as microtubule binding, is also worth considering.

The relationship between D100 and other GTP-binding proteins is, in general, limited to the composition and spacing of the three GTP-binding consensus elements (Fig. 3). However, homology of D100 with Mx1 and VPS1p extends over more than the first 700 amino acids of D100 (Fig. 5). This identifies D100, the Mx proteins and VPS1p as members of a new family of GTP-binding proteins. This family is distinct from the protein synthesis initiation and elongation factors³⁹, the tubulins⁴⁰, the signal-transducing G proteins⁴¹, and the p21^{ras} proteins⁴². Two other families have been proposed, one including components of the signal recognition particle^{20,21,43} and the other including proteins involved in secretion⁴⁴. The members of the new family described here are, in general, larger than the members of the other families, and contain a threonine at the first position of the GTP-binding consensus element III (as do the members of the signal recognition particle family), a position shown to be functionally important in p21^{ras} by mutational analysis⁴².

The members of the new family are extensively related in their amino-terminal domains, but differ in their lengths and carboxy-terminal domains. This relationship is similar to that between the members of the kinesin and myosin families^{45,46}, for which it has been proposed that the conserved domains confer common force-producing properties, whereas the variable domains confer functional specificity by directing binding

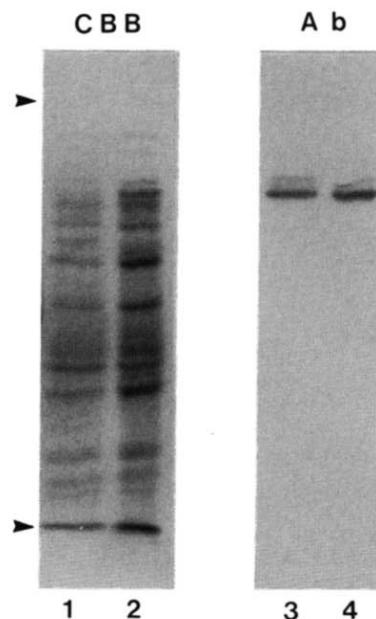


FIG. 6 Cross-reactivity of autoimmune antibody with D100 and Mx1 fusion proteins. Lanes 1, 3, Isopropyl thiogalactoside-induced plaques from clone λ ZAP-dynamin-1; lanes 2, 4, liquid lysate from isopropylthiogalactoside-induced plasmid pGEX-Mx1. The top of the blot and the dye front are marked by arrowheads. Proteins were visualized as described in the legend to Fig. 1.

METHODS. Electrobloeting and autoimmune antibody purification steps were as described in Fig. 1. Construction of pGEX-Mx1: the *Afl*III site of the 3,700 bp *Afl*III-*Eco*RI fragment of pMx34 (ref. 27) was converted to a *Bam*HI site by ligation to a double-stranded adaptor: 5'-GATCCGGCAACGAAGGTAC-CATGGGAATTC-3' and 5'-TTAAGAATTCCTACGTTGCGG-3', and the fragment was subcloned into a vector cut with *Bam*HI and *Eco*RI (pBluescript SK+), then removed as an *Nco*I-*Eco*RI fragment, using the *Nco*I site from the adaptor. This fragment was ligated to a modified plasmid, in which an *Nco*I site had been inserted between the *Bam*HI and *Eco*RI sites of pGEX-2T (ref. 55) with a double-stranded adaptor (Boehringer-Mannheim). This final construct, which contains the entire Mx1 coding sequence in-frame with a glutathione-S-transferase gene, was named pGEX-Mx1.

to different intracellular targets. Thus, a simple model predicts that D100, the Mx proteins, and VPS1p are all involved in microtubule-associated motility. How this pertains to the function of the Mx proteins is unclear. In the case of VPS1p, an involvement of microtubules can be imagined, as anti-microtubule drugs result in vacuolar fragmentation⁴⁷. We note that, despite the overall conservation of the amino-terminal domain, VPS1p contains a sequence between GTP-binding elements I and II which is absent from D100 and Mx1 (Fig. 5). According to our model, this segment could conceivably alter the interaction of VPS1p with microtubules.

In addition to the most highly conserved amino-terminal region of ~300 amino acids, sufficient similarity exists between D100, Mx1 and VPS1p that they can be readily aligned up to the carboxyl termini of Mx1 and VPS1p. Beyond this point, D100 contains a distinct carboxy-terminal extension of ~100 amino acids. This region is very proline-rich (32%) and polar (isoelectric point, pI=12.5), and is thus likely to be on the surface of the molecule in a relatively extended conformation. In view of its basicity, it may also be involved in microtubule binding. Two microtubule-binding sites are predicted for D100 from the ability of dynamin to crosslink microtubules⁵. Assuming that one microtubule-binding site lies within the conserved amino-terminal domain of D100, the other site is likely to lie at the other end of the polypeptide and could be represented by the carboxy-terminal extension. Its absence from Mx1 and

VPS1p suggests that, although these proteins are mechanistically related to dynamin, there are likely to be important functional differences as well.

Thus, analysis of the primary structure of D100 supports its biochemical identification as a component of a mechanochemical protein, and has revealed substantial homology with

a new family of proteins. The variety of contexts in which these proteins have been identified suggests that this family mediates a wide range of novel microtubule-based cellular functions.

Note added in proof: The sequence data reported will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number X54531. □

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LETTERS TO NATURE

Eötvös experiments, lunar ranging and the strong equivalence principle

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THE strong form of Einstein's equivalence principle, which dictates that gravitational binding energy will suffer the same acceleration in a uniform gravitational field as all other forms of matter and energy, can be tested by comparing the accelerations of the Earth and the Moon towards the Sun. If the Earth's gravitational binding energy, which contributes ~5 parts in 10^{10} of its total mass, does not gravitate in the same way as other kinds of mass and energy, the Moon's orbit will be distorted in a characteristic way with an amplitude large enough to be detected by lunar laser-ranging data. But as Nordtvedt¹ has pointed out, such a test is accurate only to the extent that no interfering force, such as a very feeble composition-dependent 'fifth force', fortuitously cancels a gravitational binding energy anomaly. Here we review laboratory upper limits on 'fifth forces' to set an upper limit of 1 part in 10^{12} (95% confidence level) on the Earth–Moon acceleration difference due to non-gravitational forces. Existing lunar-ranging data then verify the strong equivalence principle to an accuracy of 3%, and improved analyses of the lunar data should bring this precision down to the 0.2% level.

Einstein's equivalence principle, one of the experimental cornerstones of the general theory of relativity, has been tested with great precision in laboratory experiments of the torsion-

balance type pioneered by Eötvös. But these experiments test only the 'weak' version of the principle, because the gravitational binding energy of laboratory objects, at no more than 10^{-27} of their mass, is immeasurably small. But tests of the strong equivalence principle are now possible because the relative motion of Earth and Moon can be determined with great accuracy by measuring the round-trip travel time of light pulses emitted by Earth-based lasers and reflected back to Earth by corner cubes placed on the Moon by the Apollo astronauts. Such lunar-ranging measurements have been carried out during the past two decades at several observatories, but require careful analysis to correct for a number of possible systematic errors. Analyses^{2,3} of the lunar-ranging data obtained up to 1976 established that the accelerations of Moon and Earth toward the Sun are identical to within 14 parts in 10^{12} (95% confidence level), in agreement with the strong equivalence principle and with the predictions of general relativity. It is expected¹ that refined analyses including more recent data will test this equality to a precision of ~1 part in 10^{12} .

To what accuracy can we be sure that some other interaction, a fifth force, does not affect the differential acceleration of the Earth and Moon, and mask a violation of the strong equivalence principle? It is necessary to consider both forces mediated by vector (spin = 1 $\hbar$) and scalar (spin = 0) particles. Either will generate a potential between two point objects a distance r apart of the form

$$V_{12}(r) = \pm \frac{g_5^2}{4\pi} (q_5)_1 (q_5)_2 \frac{e^{-r/\lambda}}{r} \quad (1)$$

where g_5 is the coupling strength (the + and – signs refer to vector and scalar interactions respectively), λ is the interaction range, and q_5 is the test body 'charge', determined by the particular form of the interaction. Such an interaction would

cause the Earth and Moon to fall toward the Sun with accelerations a that differ by

$$\frac{\Delta a}{g_{\text{Sun}}} = \mp \frac{g_s^2}{4\pi G} (q_s/m)_{\odot} \Delta(q_s/m) e^{-R/\lambda} \left(1 + \frac{R}{\lambda}\right) \quad (2)$$

where g_{Sun} is the mean gravitational acceleration of the Earth toward the Sun, G is the newtonian gravitational constant, R is the mean radius of the Earth's orbit around the Sun, $(q_s/m)_{\odot}$ is the 'charge'-to-mass ratio of the Sun, and $\Delta(q_s/m)$ is the difference in 'charge'-to-mass ratios of the Earth and Moon. (For simplicity, we assume that λ is large compared to the radius of the Sun.)

Any such interaction affecting the Earth-Moon differential acceleration would also affect the differential acceleration of laboratory-sized objects, and thus could be detected by torsion-balance experiments used to test the weak equivalence principle. In essence, these experiments compare the accelerations of two different test bodies placed in a gravitational field. If all extraneous forces on the test bodies are eliminated and if the equivalence principle holds, then these accelerations will be identical, regardless of the material of which the test body is constructed. On the other hand, a 'fifth force' will produce an effect that looks like a violation of the equivalence principle. The three most precise such tests are the experiments by Dicke *et al.*⁴ and Braginsky and Panov⁵ (which compared the acceleration of Al and Au, and Al and Pt toward the Sun, respectively) and by the Eöt-Wash group⁶ (which compared the acceleration of Be and Cu and of Be and Al toward the Earth). All of these experiments obtained a null result, consistent with the weak equivalence principle, and each sets upper bounds on possible interactions affecting the Earth-Moon-Sun system.

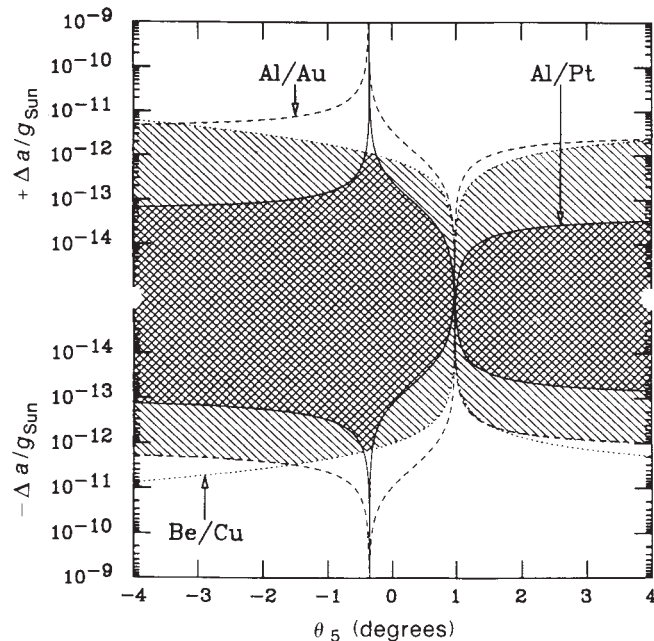


FIG. 1 Maximum allowed contribution (at 2σ) of an infinite-ranged vector force to the Earth-Moon differential acceleration toward the Sun. The lightly shaded region is consistent with the Princeton and Eöt-Wash results; the heavily shaded area is consistent with the Moscow and Eöt-Wash data. The zero occurs because the Earth-Moon charge difference vanishes at $\theta_5 \approx 1^\circ$. The poles occur at values of θ_5 where the charge difference vanishes for the Al/Au and Al/Pt test body pairs used in the Princeton and Moscow experiments. Outside the range of this plot, the constraints from the solar experiments are nearly independent of θ_5 : the Eöt-Wash terrestrial result has poles where the test-body charge difference vanishes (at $\theta_5 \approx -11^\circ$ for Be/Cu) and where the Earth's charge vanishes (at $\theta_5 \approx -64^\circ$), and a zero (at $\theta_5 \approx -49^\circ$) where the charge of the Sun vanishes. The joint constraints for $|\theta_5| > 4^\circ$ are at least as stringent as those shown in this plot.

To extract these bounds one must consider the possible charges that appear in equations (1) and (2). The most general charge for a vector interaction of stable electrically neutral matter is

$$q_s = B \cos \theta_s + L \sin \theta_s \quad (3)$$

where B and L are the numbers of baryons and leptons, respectively, and θ_s is an arbitrary parameter that specifies the precise form of the charge. It is more difficult to specify the scalar charge of a material. Although the scalar couplings may be simple in terms of the fundamental fields (for example, the expectation value of the total number of quarks and anti-quarks), the total scalar charge of a neutral atom reflects its complex structure and will depend in a complicated fashion on the atomic and nuclear binding energies. Following the ideas of Peccei, Sola and Wetterich⁷, we characterize the scalar charge with the phenomenological expression

$$q_s \approx \mu + \varepsilon_B |B| + \varepsilon_L |L| \quad (4)$$

where μ is the mass in atomic mass units (a.m.u.) and $|\varepsilon_B|$ and $|\varepsilon_L|$ are both much less than one.

For our purposes, the scalar and vector charges are very similar. In both cases a fifth force will produce an acceleration difference that is linear in $\Delta B/\mu$ and $\Delta L/\mu$ (the difference in baryons/a.m.u. or leptons/a.m.u. of the Earth and Moon, or of the two test bodies in a torsion balance). Because any given pair of test bodies may accidentally have a vanishing charge difference, at least two laboratory Eöt-Wash experiments with quite different test bodies are required to rule out the possibility that a non-gravitational force affects the Earth-Moon differential acceleration toward the Sun. The Princeton and Moscow experiments have very similar test bodies and, in this respect, are not really independent. By combining their Al/Au and Al/Pt results with Eöt-Wash Be/Cu and Be/Al data, however, we can place firm upper limits on any Earth-Moon differential acceleration owing to composition-dependent forces. We discuss the case of a vector interaction (which must couple to a linear combination of B and L) in detail. Using accepted values for the elemental compositions of the Sun⁸, Earth^{9,10} and Moon¹¹, we deduce

$$(B/\mu)_{\text{Earth}} = 1.000759(10) \quad (L/\mu)_{\text{Earth}} = 0.4869(10) \quad (5)$$

$$(B/\mu)_{\text{Moon}} = 1.000603(15) \quad (L/\mu)_{\text{Moon}} = 0.4942(10) \quad (6)$$

$$(B/\mu)_{\text{Sun}} = 0.9942 \quad (L/\mu)_{\text{Sun}} = 0.8592 \quad (7)$$

and calculate, as a function of θ_s , the maximum differential acceleration of the Earth and Moon allowed by the Princeton, Moscow and Eöt-Wash results. (The errors in equation (5) and equation (6) come from assuming that the Fe contents of the Earth and Moon are known to 10% and 30%, respectively. It is the difference in the Fe content that largely accounts for $\Delta B/\mu$ and $\Delta L/\mu$ for the Earth-Moon system. The only effect of these uncertainties is to shift the zero in Fig. 1 by a fraction of a degree, and to increase the uncertainty in Δa by $\sim 20\%$, which is included in the limits shown in Fig. 1. No errors are quoted for the Sun because these (small) errors have a negligible effect on our conclusions. The results for a vector interaction are shown in Fig. 1. At two standard deviations, the largest $\Delta a/g_{\text{Sun}} = \Delta m/m$ consistent with our results and those of the Princeton or Moscow groups is $\sim 5 \times 10^{-12}$ or $\sim 1 \times 10^{-12}$, respectively. The corresponding constraints for a scalar force are essentially identical to those discussed here for a vector interaction. This limit on the differences between fifth-force accelerations is equal to the expected precision with which the Earth-Moon differential acceleration can ultimately be extracted from existing lunar-ranging data, and is a factor of ~ 14 less than the uncertainties in existing analyses of this quantity.

As gravitational binding energy makes a 5.1×10^{-10} contribution to the Earth's mass (our result differs slightly from the value 4.6×10^{-10} quoted in refs 1 and 2 because we use a realistic model for the Earth's density rather than assuming it to be

constant), our upper limit on non-gravitational-acceleration differences coupled with the 1976 lunar-ranging analysis unambiguously establishes that gravitational binding energy has the same acceleration in a gravitational field as other forms of matter and energy to a precision of 3% (95% confidence level), and will allow the anticipated improvements in the lunar-ranging analysis to test the strong equivalence principle to a precision of 0.2%. The Eöt-Wash results⁶ can probably be improved by a factor of ~ 10 by better seismic and thermal isolation of the torsion balance. At this point one could, by combining the Eöt-Wash results with the existing Moscow data, detect non-gravitational Earth-Moon acceleration differences with a sensitivity of $\sim 10^{-13} g_{\text{Sun}}$, which lies below the anticipated precision of the lunar-ranging data. This may provide further motivation for future space experiments to test post-newtonian gravitational effects. $\square$

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Synthesis of the defect perovskite series $\text{LaCuO}_{3-\delta}$ with copper valence varying from 2+ to 3+

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MANY important questions about the origin of superconductivity in the high-temperature copper oxide superconductors could be addressed by the study of a copper oxide compound exhibiting a wide range of oxygen stoichiometry while maintaining the basic perovskite structure. A number of compounds are known in which copper occurs formally as Cu^{3+} (refs 1, 2), but most are structurally unrelated to the superconductors, so that a direct comparison of their chemical and physical properties is not appropriate. Here we report the synthesis, structure and preliminary characterization of a new oxygen-deficient perovskite series, $\text{LaCuO}_{3-\delta}$. Although superconductivity has not been observed in this system, its oxygen stoichiometry range, $0.0 < \delta < 0.5$, is the widest yet found in a copper oxide compound, allowing the formal copper valence to be varied from 2+ to 3+. This series may thus serve as a model system with which to address such questions as the nature of the Cu/O hole states in the superconductors, and the way in which magnetic and transport properties vary with carrier concentration.

LaCuO_3 was first prepared by Demazeau *et al.*³ at 900 °C and 65 kbar using KClO_3 as an oxygen buffer. Its X-ray powder diffraction pattern was successfully indexed as a rhombohedrally distorted perovskite cell and it was reported to be fully

stoichiometric and to display metallic properties down to 77 K. Because of the very high pressure and other complexities of its synthesis, however, there has been no further characterization of this important phase. In searching for milder synthetic conditions, we discovered a new series of oxygen-defect $\text{LaCuO}_{3-\delta}$ perovskites, with $0 \leq \delta \leq 0.5$.

Phases in the $\text{LaCuO}_{3-\delta}$ system were prepared at 900 °C at oxygen pressures of 0.2–1 kbar using coprecipitated La and Cu hydroxide precursors. The precursors were decomposed by slow heating to 650 °C under oxygen before reacting them in high-pressure oxygen. Exclusion of CO_2 and other organic impurities from the starting materials and an oxidizing atmosphere was necessary to obtain single-phase, homogeneously oxidized products. Compositions were determined by thermogravimetric analyses with an accuracy in δ of ± 0.02 . High-resolution X-ray powder diffraction data for the various phases in the $\text{LaCuO}_{3-\delta}$ system were collected at the Brookhaven National Synchrotron Light Source. Structural refinements were performed with a modified Rietveld program (S. La P., J. B., R. B., D. C. and B. S., manuscript in preparation).

The phase and crystallographic data for $\text{LaCuO}_{3-\delta}$ are summarized in Fig. 1. These phase fields were obtained from samples prepared directly, or modified by reduction of a $\delta = 0.1$ composition in N_2 or O_2 (1 atm) at 300–750 °C. Thus Fig. 1 is not a true phase diagram, because the data were not obtained under equilibrium conditions. We have found that it is possible to change the ranges somewhat by varying the preparation or processing conditions. The evolution of structure as δ is varied is given in Fig. 2. We observe three distinct ordered arrangements of oxygen (or vacancies). A tetragonal perovskite with a homogeneity range $0 \leq \delta \leq 0.2$ occurs at 900 °C and $P_{\text{O}_2} = 0.4$ –1 kbar. Samples for which $\delta = 0$ were obtained for $P_{\text{O}_2} \geq 0.5$ kbar, but in general, oxygen stoichiometries were dependent on several factors, including the homogeneity and purity of the starting materials. At lower oxygen pressures, $P_{\text{O}_2} = 0.2$ –0.4 kbar, the degree of oxidation decreases, leading to the formation of a monoclinic phase stable over the interval $0.2 \leq \delta \leq 0.4$. Both forms are metallic, with room-temperature resistivities of $100 \mu\Omega \text{ cm}$ and

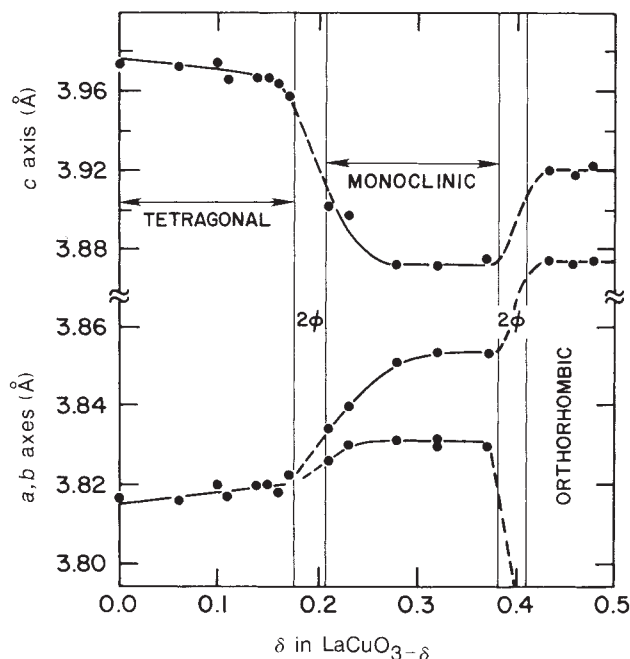


FIG. 1 The variation of the perovskite subcell parameters with oxygen content in $\text{LaCuO}_{3-\delta}$. The subcell a axis of the orthorhombic phase is 3.70 Å . 2ϕ , approximate two-phase region discussed in the text.

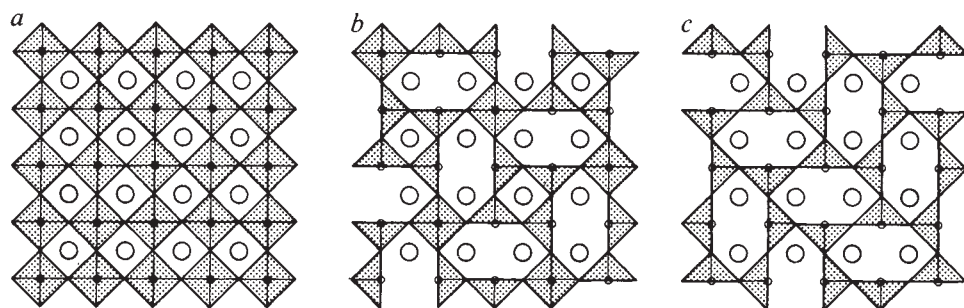


FIG. 2 The evolution of oxygen-vacancy ordering in the $\text{LaCuO}_{3-\delta}$ perovskite system. The structures shown are the (001) plane projections for: *a*, the tetragonal phase with $\delta=0$; *b*, the idealized monoclinic perovskite with $\delta=0.4$; *c*, the idealized orthorhombic perovskite with $\delta=0.5$. La, large open circles; Cu-O polyhedra, shaded.

300 $\mu\Omega$ cm for $\delta=0.14$ (tetragonal) and $\delta=0.33$ (monoclinic), respectively. Superconductivity has not been observed in either case down to 4.2 K. Lastly, an orthorhombic perovskite can be prepared by heating the tetragonal form to 700–800 °C in N_2 . This phase has a homogeneity range between $0.43 \leq \delta \leq 0.5$ and is an insulator.

The powder X-ray diffraction profiles of the three phases observed in this system are shown in Fig. 3. The tetragonal perovskite with $0.0 \leq \delta \leq 0.2$ exhibits the simplest diffraction pattern with no observable supercell reflections. The diffraction pattern of the tetragonal $\delta=0$ phase is readily distinguishable from the rhombohedral form³. All three of the phases contain this basic perovskite pattern, but as oxygen vacancies are created ($\delta > 0.2$) supercell reflections begin to appear (Fig. 3*b*). In the monoclinic phase, these are related to the perovskite subcell by approximately $\sqrt{5}a \times a \times \sqrt{5}a$. The diffraction pattern of the third phase (Fig. 3*c*) can be indexed using an orthorhombic cell with lattice parameters related to those of the perovskite subcell by approximately $\sqrt{2}a \times 2\sqrt{2}a \times a$. As is apparent in Fig. 3, the samples are virtually free of secondary phases and in the best cases, no impurity phases could be detected to the limit of our diffractometer ($\sim 3\%$) or in the synchrotron data ($\sim 0.5\%$).

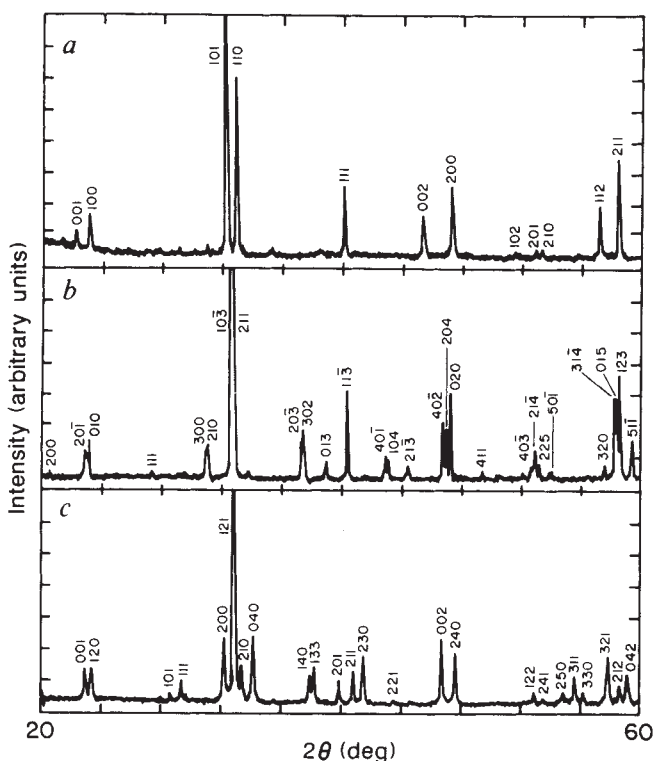


FIG. 3 The powder X-ray diffraction profiles in the region $2\theta=20$ – 60° of the three phases observed in the $\text{LaCuO}_{3-\delta}$ system, with Miller indices indicated. *a*, tetragonal perovskite with $\delta=0$; *b*, monoclinic perovskite with $\delta=0.33$; *c*, orthorhombic perovskite with $\delta=0.5$. For clarity, not all of the Miller indices are shown for the monoclinic phase.

The structure of the $\delta \approx 0$ tetragonal phase (Fig. 2*a*), is a simple tetragonal distortion of a cubic perovskite with $a = 3.81875(5)$ Å and $c = 3.97270(7)$ Å, space group $P4/m$. This results from a slight Jahn-Teller-like elongation of the CuO_6 octahedra, in which there are four in-plane Cu–O bonds at 1.909 Å, and two apical Cu–O bonds at 1.986 Å. This is in contrast to the rhombohedral form in which all Cu–O distances are equal at 1.94 Å (ref. 3). The unit-cell volume of the tetragonal phase is $\sim 5\%$ larger than that of the rhombohedral form³, which suggests that the latter is the high-pressure and the former the low-pressure polymorph of LaCuO_3 . Webb *et al.*^{4,5} have reported that a tetragonal phase of LaCuO_3 with lattice parameters $a = 5.431$ Å and $c = 7.84$ Å can be prepared by heating the rhombohedral form to 408 °C. Not all of the lines in the powder pattern of this material could be accounted for, however, and there was in general a very poor fit of the observed and calculated lines. Furthermore, no attempt was made to measure the oxygen content and it seems likely that this material may be non-stoichiometric, because one would expect a Cu^{3+} material to evolve oxygen at this temperature. There are no obvious similarities between the reported diffraction lines and those of the tetragonal phase of LaCuO_3 reported here.

Below the phase field of the tetragonal form, oxygen-vacancy ordering leads to a monoclinic perovskite (Fig. 2*b*). The vacancies order along pseudo-cubic $\{201\}$ axes, giving rise to a new $\sqrt{5}a_p \times a \times \sqrt{5}a_p$ cell with lattice parameters $a = 8.62884(5)$ Å, $b = 3.83076(2)$ Å and $c = 8.65149(5)$ Å for $\delta=0.33$. The synchrotron diffraction peaks of this phase are extremely sharp ($\sim 0.025^\circ$ full width at half maximum) so that the monoclinic distortion from orthorhombic symmetry can be readily distinguished, although it is very small ($\beta = 90.214(4)^\circ$). For the expanded cell the unit-cell composition is $\text{La}_5\text{Cu}_5\text{O}_{13.35}$. The structure of this phase closely resembles that of $\text{BaLa}_4\text{Cu}_5\text{O}_{13+x}$ (ref. 6), although the latter is tetragonal. It comprises one-dimensional chains of CuO_6 octahedra oriented along b which share corners with four CuO_5 square pyramids in the (010) planes. The CuO_5 pyramids share corners with four other square pyramids but are oriented with their basal faces at 90° to one another. In contrast to $\text{BaLa}_4\text{Cu}_5\text{O}_{13}$, the non-idealized structure (S. La P., J. B., R. B., D. C. and B. S., manuscript in preparation) is significantly distorted from tetragonal symmetry, and the Cu–O–Cu bonds adjoining octahedra and pyramids are bent considerably below the ideal bond angle of 180° .

The third phase observed in the $\text{LaCuO}_{3-\delta}$ system appears at $\delta=0.43$. Rietveld refinement shows that oxygen-vacancy ordering in the (001) plane, which is complete at $\delta=0.5$, creates a cell with lattice parameters related to those of the pseudo-cubic perovskite by $a \approx \sqrt{2}a_p$, $b \approx 2\sqrt{2}a_p$ and $c \approx a_p$. Cell parameters obtained from the refinement are $a = 5.5491(1)$ Å, $b = 10.4782(2)$ Å and $c = 3.87956(7)$ Å for a sample of composition $\text{LaCuO}_{2.53}$. The idealized structure shown in Fig. 2*c* is isostructural with $\text{CaMnO}_{2.5}$ (ref. 7) and is entirely composed of corner-shared CuO_5 pyramids which form pairs by sharing oxygen vacancies in the (001) planes.

As oxygen is removed from the tetragonal $\delta=0$ phase, two CuO_5 pyramids and two sites that are formally Cu^{2+} are created in the form of square-pyramidal pairs for each vacancy inserted.

The result is that Cu is found only in octahedral and/or square-pyramidal coordination throughout the homogeneity range of $\text{LaCuO}_{3-\delta}$, and that the number of holes (Cu^{3+} sites) will be formally equal to the number of octahedral coordination sites in the system. This aspect of the structure means that the 3+ and 2+ valence states of Cu in $\text{LaCuO}_{3-\delta}$ are 'tagged', a feature which can be used to great advantage in unravelling the electronic structure of the cuprates. For example, we have exploited this to resolve the mystery of the 'missing' electron paramagnetic resonance in the superconducting cuprates^{8,9}. As a result of these features and of the wide range of copper valence attainable in this system, this may be an ideal system with which to probe the subtle interplay of crystal-chemical and physical properties, to examine and compare the normal-state properties of the superconducting and related cuprates, and to study the evolution of the electronic properties at higher carrier concentrations. A detailed study of the effects of systematic valence changes on the electrical and magnetic properties in the $\text{LaCuO}_{3-\delta}$ system will be reported shortly. $\square$

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Large bubble-like features ordered on a macrolattice in helium-implanted gold

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IMPLANTATION of metals at temperatures near ambient with helium ions can result in the formation of an ordered array—a superlattice—of small (~ 2 nm in diameter) helium bubbles^{1–11}. The superlattice has the same symmetry as the atomic lattice of the metal but with a lattice spacing about 20 times greater. We now report the observation, in helium-implanted gold, of an ordered array—a 'macrolattice'—of artefacts ~ 60 nm in diameter, which we believe also to be helium bubbles. Again, the macrolattice has the same symmetry as the gold lattice, but the lattice spacing is about 400 times larger. The coexistence in a single specimen of three separate lattices of very similar structure but which differ so markedly in scale provides a new perspective on ordering phenomena. In particular, an understanding of the processes that drive bubble ordering may provide more general insight into the development of microstructures in metals during ion irradiation¹². Such information is of value for the production of wear- and corrosion-resistant surfaces by ion implantation^{13,14} and the development of materials with appropriate erosion and gas-loading properties for use in future nuclear-fusion reactors^{15,16}.

To produce a bubble superlattice the helium is implanted in the metal at a temperature $<0.3 T_m$, where T_m is the melting temperature of the metal. For implantation at these low temperatures the bubbles are overpressured^{15,17}, in the sense that the surface tension of the metal alone is not sufficient to contain the gas pressure. As a consequence, the crystal lattice surround-

ing each bubble is under considerable stress. In most studies the helium energy is ~ 30 keV with the ion beam incident along the normal to the target surface. Under these conditions, the helium concentration initially increases rapidly with depth, reaching a maximum at a depth ~ 100 nm; thereafter the concentration falls rapidly. Thin sections, typically ~ 60 nm thick, are taken from the implanted surface by thinning the foil to perforation from the rear surface ('back thinning') using chemical electropolishing. In face-centred-cubic metals, the bubble lattice has the same symmetry as the atomic lattice of the host metal and a lattice constant of 6–8 nm. Typical bubble diameters are ~ 2 nm. A high-magnification transmission electron micrograph of helium bubbles in copper, the most extensively studied of the face-centred-cubic metals, is shown in Fig. 1. It is rarely possible to image the very small superlattice bubbles so clearly in the presence of the high levels of gas, strain and damage in the implanted layer. But even in cases where the bright-field micrographs are ambiguous, the occurrence of satellite reflections around matrix spots in electron diffraction patterns can often provide conclusive evidence of superlattice formation⁴.

A previous study showed that a bubble superlattice could be formed in gold⁶ and this work is a continuation of that study. To obtain a more uniform helium concentration extending deeper into the target, the helium was implanted at higher energy (160 keV) and at an oblique angle (60° to the surface normal). The helium was broadly distributed about a maximum concentration located at a depth of ~ 250 nm. Thin sections were prepared by backthinning using argon-ion-beam milling. A gas-bubble superlattice was identified at high magnification in a transmission electron microscope (Philips EM 420 ST). The micrographs and diffraction patterns obtained (not of sufficient quality to be reproduced usefully here) enabled the superlattice parameters to be determined. The average bubble diameter of ~ 2 nm and lattice constant of ~ 8 nm are consistent with those found previously⁶. It was at low magnification, however, that the new features were discovered.

The structure in these same gold specimens is dominated by a mesh-like array (Fig. 2), suggestive of a three-dimensional lattice with a periodicity much larger than that of the superlattice. It is made up of rows of artefacts in the two $\langle 220 \rangle$ directions that are normal to the direction of the electron beam. We believe that the artefacts defining the mesh are bubbles because: (1) the images have the appearance of large bubbles under a variety of imaging conditions including imaging through the thicker regions of specimens in a 300-keV microscope, (2) individual

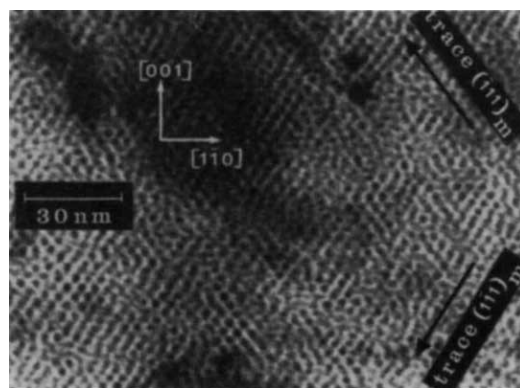


FIG. 1 A transmission electron micrograph of helium gas bubbles in copper following implantation with 4×10^{21} 30-keV He^+ m^{-2} at 300 K. The electron beam is along $[110]$ in the atomic lattice of the copper and is normal to the plane of the bubble layer and specimen surface. The bubbles are ordered on a superlattice. Principal directions of the copper lattice are indicated. The orientation of bubble rows relative to these directions forms part of the evidence that the bubble superlattice has the same symmetry as the metal lattice.

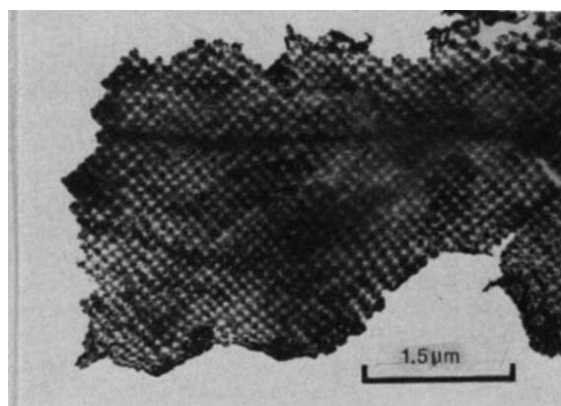


FIG. 2 A transmission electron micrograph of artefacts defining a two-dimensional mesh, in a (100) gold grain following implantation with 1×10^{22} 160-keV $\text{He}^+ \text{m}^{-2}$ at 280 K. The electron beam is along [100] in the atomic lattice of the gold. Although the artefacts when imaged (as here in the thinnest regions of the specimen) do not have the appearance of bubbles, a variety of observations (see text) have led to the conclusion that they are the remnants of large helium bubbles arranged on a macrolattice.

artefacts in dark-field images have a bubble-like shape defined by thickness extinction contours (approximately circular) of different radii, (3) there are patches in which all the artefacts are larger than those in the surrounding regions (Fig. 3). This is readily explained if the artefacts are bubbles and difficult to explain if they are not. The pressure of the gas in the cavity of an overpressured bubble exerts a large stress on the surrounding metal. Suppose a particular bubble, because of favourable local conditions, were to grow at a rate faster than the average and cause a doming of the specimen surface. A component of tensile stress in the direction of the surface normal would be imposed on the matrix surrounding each of the neighbouring bubbles—a stress which would be expected to enhance bubble growth^{18,19}. The correlated growth of bubbles in a patch caused by this mechanism could be described in terms of a lowering of the elastic energy resulting from the group of bubbles cooperating to distort the surface. Cooperative growth of this kind is expected

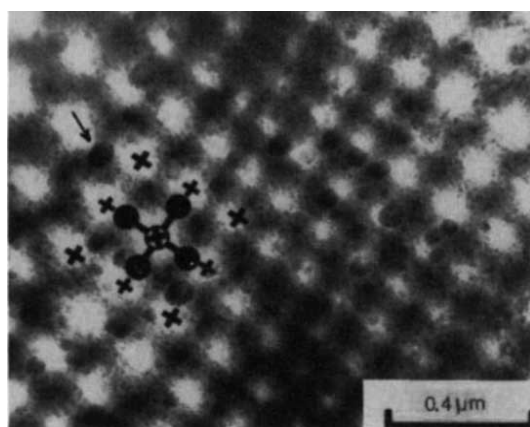


FIG. 3 A direct transmission electron micrograph showing patches in which all the bubble-like artefacts are larger than in the surrounding regions. Except for such isolated patches the size and spacing of artefacts is remarkably uniform throughout the specimen with typical diameters of ~ 70 nm and nearest-neighbour separations of ~ 105 nm (measured along $\langle 220 \rangle$). The grid (solid black circles and lines) shows the typical spacing of artefacts in the surrounding regions. The correlated growth of artefacts in a patch forms part of the evidence that the artefacts are helium bubbles. The disk-like images, such as that indicated by the arrow, are attributed to pieces of metal blown out from the surface by the bursting of large bubbles during ion-beam thinning.

only for large bubbles lying close to the surface. For small bubbles deep in the metal, the interaction between bubbles may act to inhibit bubble growth⁹.

Typical bubbles have a diameter of ~ 70 nm and a spacing of ~ 105 nm measured along $\langle 220 \rangle$ rows. The spacing is a factor of about 20 times greater than that found for the bubble superlattice in gold⁶ and is approximately twice the spacing of the coarsest void lattice that has been produced by ion irradiation in a pure (single element) face-centred-cubic metal. (Void lattices with large spacings are produced by irradiation²⁰ at elevated temperatures $\sim 0.4 T_m$). This is the first observation of bubble ordering on such a large scale. The result is surprising not only because of the large spacing but also because the macrolattice has a high degree of perfection, extends throughout the specimen and was formed in heavily implanted metal at low temperature ($\sim 0.22 T_m$) where vacancies have very limited mobility. The close-packed directions in the bubble macrolattice are parallel to the close-packed directions, $\langle 220 \rangle$, of the gold atoms in the (100) surface plane. The specimen thickness is estimated to be ≤ 100 nm and this allows just one (100) bubble plane to be contained in the depth of the specimen. For a single plane of bubbles, the directions of the bubble rows are consistent with the macrolattice having a face-centred-cubic symmetry and an alignment parallel with the atomic lattice of the gold. The small bubbles forming the superlattice are in the metal between this single plane of large bubbles and the specimen surfaces and also in the metal columns between neighbouring large bubbles (see Fig. 4). The two bubble lattices thus coexist in the atomic lattice of the host metal. The single plane of large bubbles may be a remnant of a fully ordered three-dimensional bubble macrolattice present in the target before thinning.

The discovery of a bubble macrolattice increases the experimental knowledge with which the various theories of bubble ordering should be consistent. There are three classes of such theories involving: (1) elastic interaction between bubbles²²⁻²⁴, (2) planar diffusion of host interstitial atoms^{25,26} and, (3) interaction between dislocations 'punched out' from overpressured bubbles^{9,27-29}. Although these mechanisms are complex and each of the theories has both advantages and disadvantages (see, for

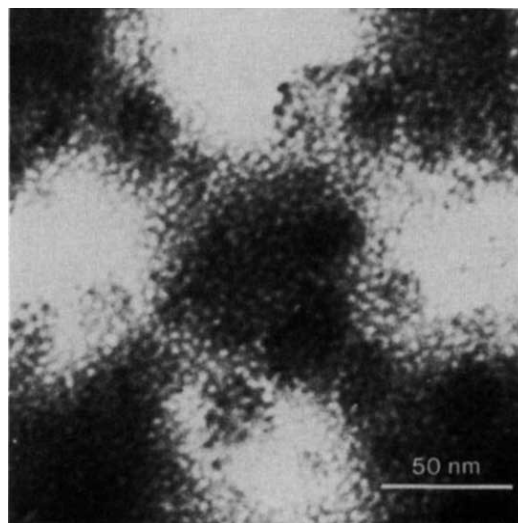


FIG. 4 A high-magnification transmission electron micrograph (under-focus contrast) showing the small bubbles (~ 2 nm in diameter) in the metal between the large bubbles that lie on the macrolattice. The four light areas are the positions of large bubbles that have intersected the specimen surface. The large bubbles have distorted the surrounding metal and perturbed the alignment of the bubble superlattice, but traces of bubble alignment can still be seen. The ordering of the small bubbles can be made much more obvious by carefully removing the remnants of the large bubbles by electropolishing.

example, ref. 30) precluding a detailed analysis here, some preliminary comments can be made.

The ratio of lattice parameter to bubble radius for the macro-lattice is about four. This value is in agreement with treatments^{22,23} based on the ordering of spherical cavities owing to elastic interactions. It is not yet clear, however, that elastic interactions are sufficiently strong to explain bubble ordering³⁰. Despite imperfections, such as macrolattice 'dislocations' formed by terminating bubble rows, there is a clear long-range character to the ordering in the plane of the implanted layer. There seems to be no ready explanation for this feature in terms of the theories based on the planar diffusion of metal atoms. For example, it seems improbable that the mean free path for the lateral diffusion of gold atoms in a metal matrix containing high levels of gas and damage could be large enough to explain the long-range ordering.

The dislocation-punching theories of bubble ordering are based on the assumption that there is a strong mutual repulsion acting between neighbouring bubbles. This repulsion is in addition to the attractions and repulsions proposed in earlier theories^{21,22}. The mutual repulsion was attributed to a repulsion between dislocation loops of diameter comparable with that of the bubbles. (Here, a dislocation loop can be thought of as a circular planar disk of atoms, one atom thick, pushed out from the surface of the bubble into an interstitial position between adjacent dense-packed atom planes of the host metal.) These loops were postulated to lie in the metal separating nearest-neighbour bubbles²⁸. In the case of the superlattice such dislocations have never been observed. This led to the proposal that the mutual repulsion might result from strong compressional stresses set up in the metal between bubbles by the tendency of overpressured bubbles to punch dislocations rather than from the presence of dislocations⁹⁻¹¹. It should be possible to image dislocations of a size comparable with the diameter of the large bubbles forming the macrolattice but we have found no evidence of such dislocations. This supports the proposal that the interaction between bubbles may need to be modelled, not in terms of existing dislocations, but rather in terms of the compressional stress in the metal set up between neighbouring bubbles⁹⁻¹¹. □

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New pressure-induced transformations of silica at room temperature

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SILICA has many polymorphs, and the solid-state transformations between these under pressure have been studied extensively to gain insight into the mechanisms of such high-pressure transitions in general. Most of the transformations of silica polymorphs have been thought to require high temperatures as well as high pressures. Recently, however, a pressure-induced change in silicon coordination in silica glass¹ and amorphization of quartz and coesite (a high-pressure phase)² have both been observed at room temperature. Here we report two new room-temperature pressure-induced transformations of silica, observed by *in situ* X-ray diffraction. At pressures above 10 GPa, cristobalite transforms into a new phase, and above 30 GPa a second new phase is formed, both of which are crystalline. Above 60 GPa, however, quartz transforms to poorly crystallized stishovite, probably via an amorphous state. The new high-pressure polymorphs are probably metastable, and may involve a change in silicon coordination.

The crystal chemistry of silicate melts and glasses indicates that pressure-induced reversible changes between four-fold and six-fold Si coordination may occur under pressure³. Such changes in coordination affect the melt and glass density and viscosity, and could enhance the processes of chemical differentiation with depth in the Earth's mantle⁴. Cristobalite is a high-temperature polymorph of SiO₂ with a very loose structure. A molecular dynamics calculation predicted⁵ that at 16.5 GPa cristobalite may transform into a metastable structure of space group *Cmcm*, containing both four-fold and six-fold Si coordination. This phase was predicted to transform into stishovite at 23 GPa. Our experiments were performed to test these predictions. The details of these experiments are very similar to our previous study on SiO₂ up to 120 GPa (ref. 6). To avoid the effect of moisture, finely powdered samples of synthetic cristobalite and quartz were dried at 100 °C for 2 h and embedded in a small hole (100 µm) in a stainless-steel

TABLE 1 X-ray diffraction data for SiO₂

Phase	Pressure (GPa)	d spacing (Å)	Intensity
XI	15.1	3.098	S
		2.883	VW
		2.702	VS
		2.373	M
		2.180	W
		2.026	M
XII	53.1	2.493	W
		2.114	VW
		1.917	M
		1.859	M
XIII	0.0*	3.166	W
		2.966	VW
		2.595	S
		2.244	VW
		1.972	W
		1.933	W
		1.834	VW
		1.494	M

Starting material was cristobalite. S, strong; M, medium; W, weak; VS, very strong; VW, very weak.

* Quench product recovered from 53.1 GPa.

gasket. Pressure in the sample chamber was measured using ruby fluorescence⁷. Three or four ruby chips were spread in the sample chamber and concentric distributions of pressure were observed. Average pressures as well as maximum and minimum pressures in the area irradiated by X-rays (70 μm across) were used for the analysis. The *in situ* X-ray observations of the samples at high pressure and ambient temperature were made using a rotating-anode high-power X-ray source operated at 55 kV and 160 mA. Filtered Mo K α radiation was collimated by a 60- μm pin hole and the diffracted X-rays were measured by a Debye-Scherrer camera with a radius of 57.3 mm. The typical exposure time was three days. X-ray diffraction observations were also made of recovered samples at ambient temperature and pressure. To determine the stable phase, samples mixed with a small amount of platinum black (which absorbs Nd-YAG laser radiation) were heated, at the desired pressure, by the laser radiation, and examined by *in situ* X-ray diffraction.

Results of the room-temperature compression of cristobalite are shown in Fig. 1. Because of its loose structure, cristobalite is much more compressible than the other polymorphs of silica. The zero-pressure bulk modulus, K_0 , calculated from the X-ray diffraction data using a Birch-Murnaghan equation of state is 18 ± 1 GPa, very close to the value of 17.2 GPa obtained by molecular dynamics calculations⁸. This value is more than one order of magnitude smaller than that of stishovite. A similar bulk modulus, $K_0 = 15.7$ GPa, was obtained in another X-ray diffraction study⁹.

At 10 GPa and room temperature, cristobalite transforms into a new phase XI that is different from stishovite. None of the known polymorphs of silica can account for the observed diffraction pattern (Table 1) and the structure of this new phase has not yet been determined. This phase is unquenchable and on release of pressure, it transforms back into cristobalite. Molecular dynamics simulations⁵ have also predicted an unquenchable *Cmcm* phase, but the diffraction pattern of our high-pressure phase is different to that expected from the simulation phase, so it is not clear whether the two are related.

Further compression produces another new phase, XII. The X-ray diffraction lines of this phase are weak and broad, however, making analysis of the structure very difficult. This phase is also different from the known polymorphs of silica. On the release of pressure, it transforms into another new phase, XIII, different from cristobalite and other polymorphs of silica (Table 1). When the XI phase was heated above 1,000 °C at

TABLE 2 Products of room-temperature compression of quartz

Run	407	421	725	702	921
Pressure (GPa)	59* (61–56)†	69 (72–65)	72 (74–69)	62 (66–56)	70 (74–67)
Time (h)	72	72	72	1	72
High-pressure phase	rutile	rutile	rutile	‡	‡
Recovered sample at ambient condition	‡	‡	rutile	amorphous	rutile

* Average value in irradiated area (70 μm across).

† Maximum and minimum pressures in irradiated area.

‡ Not measured.

15 GPa, it transformed into a single phase of stishovite. We therefore suggest that the new phases formed by compression of cristobalite at room temperature are metastable.

The experimental results for quartz are summarized in Table 2. The transformation from quartz or coesite to stishovite had been believed to require temperatures >600 °C¹⁰, but our results indicate that stishovite can be formed even at room temperature when enough excess pressure is applied. This stishovite is poorly crystallized and has very broad and weak diffraction lines. Moreover, the observed 111 diffraction line is about twice as intense as the 110 line. On the other hand, for randomly oriented particles, the 111 line is 35% less intense than the 110 line. This implies the existence of preferred orientation in the sample.

To investigate the kinetics of the quartz–stishovite phase transformation, two experiments were carried out (Runs 702 & 921 in table 2). Strong X-ray irradiation of crystals can cause dislocations, which might accelerate the crystallization or phase transformation. To avoid this, we did not use high-pressure *in situ* X-ray diffraction in these runs. We did not find any diffraction lines from a sample recovered after holding quartz at 62 GPa for 1 h (Run 702), but we did observe weak broad stishovite lines when the same starting material was kept at 70 GPa for 3 days (Run 921). The quartz-to-stishovite transition at room temperature is therefore very sluggish.

Our results indicate that the room-temperature pressure-induced transformation from quartz to stishovite occurs through the amorphous state. This implies that the activation energy for the formation of silica polymorphs decreases dramatically under very high pressure, as well as in the low-pressure region¹¹. Williams and Jeanloz¹ suggested that a pressure-induced change in the coordination of silicon occurs at room temperature, based on the observation of an infrared absorption of silica glass. Our crystallographic evidence demonstrates that this kind of transformation can occur at room temperature. Our results are also consistent with the model proposed by Stolper and Ahrens³, which suggested a coordination change of silicon by displacement of the SiO₄ tetrahedron, without a large diffusion of atoms.

Our high-pressure experiments were performed by directly compressing powdered samples between anvils and large pressure gradients in the sample chamber were observed (Table 2). It is therefore difficult to discuss the details of the mechanism of transformation. To avoid this problem, a quasi-hydrostatic pressure-transmitting medium, such as solidified rare gas, could be used.

Cristobalite does not show pressure-induced amorphization but transforms directly into the crystalline phase. The second high-pressure phase, XII, is stable at ~ 60 GPa, whereas stishovite is formed when quartz is used as the starting material. The structure of XII has not yet been determined but we have estimated that its compressibility is very small. The value of d/d_0 for each diffraction line of XII is between 4.3×10^{-4} GPa⁻¹ and 8.7×10^{-4} GPa⁻¹ at ~ 50 GPa, almost the same as that of stishovite (the average value for the four diffraction lines is $\sim 6.8 \times 10^{-4}$ GPa⁻¹)⁶. The X-ray pattern of XII phase has no

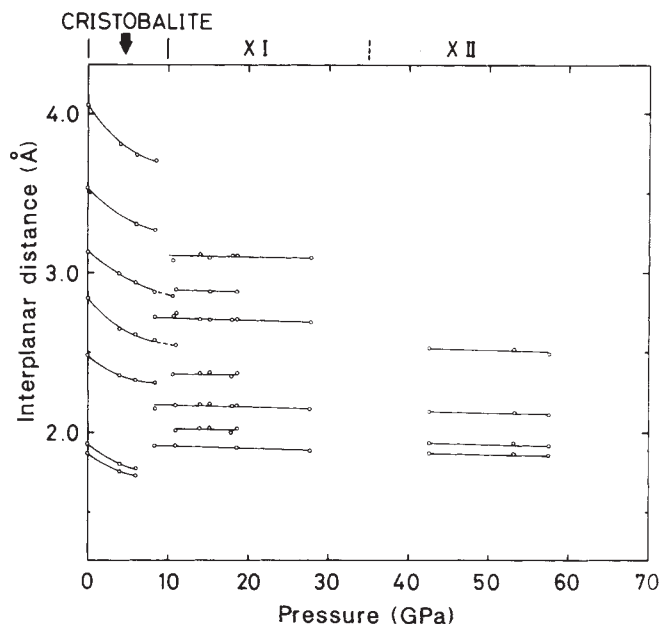


FIG. 1 Pressure dependence of interplanar spacing of cristobalite.

diffraction line corresponding to $d > 3.0$ Å. These results imply a close-packed structure.

To understand the difference between the transformations of quartz and cristobalite, we need to know the Si-O coordination number of the XII phase. Both XI and XII phases are poorly crystallized, however, and the quality of the diffraction patterns are not sufficient to solve the structures. Structural information from Raman scattering, infrared absorption and EXAFS (extended X-ray absorption fine structure) would be useful. □

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A 210,000-year record of barium variability in the deep northwest Atlantic Ocean

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THE deep ocean is the storehouse for most of the carbon and nutrients in the ocean–atmosphere system; together with its outcrops at high latitudes, the deep ocean is of great importance in driving glacial–interglacial changes in the carbon dioxide content of the atmosphere^{1–3}. The chemical and physical structure of the deep glacial oceans can be reconstructed by means of palaeochemical tracers; for example, the cadmium and carbon isotope contents of benthic foraminifera have been used to reconstruct the phosphate (or labile nutrient) compositions of deep waters^{4–14}. Recently, it was proposed¹⁵ that barium in benthic foraminifera could be used to reconstruct the distribution of refractory, deep-regenerated chemical properties in the water masses of the glacial oceans. A reconstruction of barium concentrations in the oceans at the time of the Last Glacial Maximum revealed the greatest changes in the North Atlantic, where barium concentrations in deep water were up to 50% higher than at present¹⁶. Here we present a record of the benthic Ba/Ca ratio in the deep northwest Atlantic stretching back to interglacial stage 7 (~210 kyr ago), encompassing two full glacial cycles. A comparison of the barium record with that of the other nutrient-like tracers lends confidence in the general agreement between these tracers while showing some spectral variability unique to barium, thus underscoring its utility as an additional palaeoceanographic tracer.

Piston core CHAIN 82, station 24, core 4PC was raised from 3,427 m on the western flank of the Mid-Atlantic Ridge (41°43' N, 32°51' W). The sedimentation rate averages ~3 cm kyr⁻¹, although the rate is not uniform over the length of the core. The sample depths reported here are for contiguous

samples relative to the core liner; there was a void interval between 347 and 380 cm apparently resulting from mechanical fracturing⁵.

Figure 1 illustrates the benthic foraminiferal Ba/Ca record, as well as oxygen isotope, carbon isotope and Cd/Ca records from a previous study⁵. Ba was determined on purified shells by isotope-dilution plasma mass spectrometry^{15,16}. The analytical precision of the Ba/Ca ratios is estimated to be better than 3%. Many of the samples were run in duplicate, as indicated in Table 1. Ba/Ca ratios were determined on samples of the benthic foraminifera *Uvigerina* spp. where available. Certain intervals have low to near zero *Uvigerina* spp. abundances: 0–35 cm (stage 1), 250–300 cm (stage 5a), 450–465 (stage 5e) and 650–670 cm (stage 7a–b)⁵. *Cibicides kullenbergi* was used instead of *Uvigerina* spp. between 0 and 30 cm. Calibration of these two species for uptake of Ba indicates no statistically significant difference¹⁵. Ba/Ca ratios from *Uvigerina* spp. were used in the other low-abundance intervals; these values should be interpreted with caution because small samples were used for analysis. In addition, bioturbation can bias any foraminiferal parameter measured in zones of low abundance.

The timescale for core 4PC shown in Fig. 1 is based on the accepted timing for the main features of the oxygen isotope record identifiable in this core^{5,17}. The bottom of the core (668 cm) has been assigned to the transition between stage 7a and 7b, or ~220 kyr ago. This produces a realistic sedimentation rate for stage 7 (~2 cm kyr⁻¹) and is justifiable given that the benthic oxygen isotope record never reaches the heavy values (~4‰) characteristic of stage 7b. In addition, the disappearance of *Uvigerina* spp. in the bottom 20 cm of the core is consistent with the change observed in early stage 7a in neighbouring core V30-97¹⁸.

The principal features of the Ba/Ca record show a strong covariance with the generally accepted climate cycles of the past 200,000 years^{19,20}. In general, minima in benthic Ba are associated with interglacial stages and maxima are associated with glacial stages. Interglacial stages (1, 3, 5 and 7) are characterized by Ba/Ca values of ~1.8–2.6 μmol mol⁻¹, and glacial stages (2, 4 and 6) are characterized by values of ~2.6–4 μmol mol⁻¹. The most Ba-depleted deep waters at these sites were present in stages 5a, 5c and 7, and the most Ba-enriched deep waters were present during stage 2 and 6. One might also expect stage 5e to be very low in Ba and measured values in this interval are probably not representative of the true values because of extremely low *Uvigerina* abundances⁵.

The Ba and Cd records from core 4PC are generally similar in confirming high nutrient levels during glacial periods and low nutrient levels during interglacial periods (Fig. 1). There are, however, clear differences between the two records: (1) as previously noted from a study of both Atlantic and Pacific cores¹⁶, the Ba contrast between the oceans vanishes during glacial stage 2. The high Ba values of stage 6 of core 4PC suggest the same for other glacial extrema, although this remains to be proven; (2) little direct correspondence is seen between the short-timescale fluctuations of Cd and Ba. Comparison of the Ba record with the carbon isotope record reveals that Ba/Ca maxima and δ¹³C minima correspond in stage 2, 4, 5d and in the three cycles of stage 6. A direct comparison of the carbon isotope and Ba data is misleading, however, because a large portion of ¹³C variability is due to transfer of carbon between the ocean and continental organic carbon reservoir, which is not expected to affect Ba^{21,22}.

The short residence time of barium in the ocean, estimated at ~10,000 years²³, is especially important in any consideration of its long-term variability. A survey of Ba in the oceans of the Last Glacial Maximum suggest that average Ba concentration of the ocean might have been ~15% lower¹⁶. On the 200,000-year timescale of the core 4PC record, changes in the Ba content of the oceans are quite possible. The variability recorded by the benthic foraminifera from core 4PC argues against large changes

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TABLE 1 Ba/Ca in benthic foraminifera of core 4PC

Interval (cm)	Ba/Ca ($\mu\text{mol mol}^{-1}$)	SD	<i>n</i>	<i>r</i>	Interval (cm)	Ba/Ca ($\mu\text{mol mol}^{-1}$)	SD	<i>n</i>	<i>r</i>	Interval (cm)	Ba/Ca ($\mu\text{mol mol}^{-1}$)	SD	<i>n</i>	<i>r</i>
0-2	2.33*	0.06	2		224-226.5	2.88	0.04	2		466-468	2.95	0.01	2	
4-5	2.24*	0.01	2		226.5-228.5	2.87	0.07	3		468-470	2.83	0.07	2	
8-9	2.26*		1		228.5-230	3.19		1	1	470-471	2.98		1	
12-13	2.42*	0.08	2		230-233	3.00		1		472.5-475.5	3.23	0.10	2	
19.5-20.5	2.40*		1		233-236	2.65	0.20	3		478.5-482	3.91	0.24	2	
22-24	2.38*		1		239-242	2.30	0.20	3		485.5-489	4.17		1	
25-26	2.72*		1		241.5-243	1.96		1		490-492	3.74	0.09	2	
35-36	2.51		1		243-246	1.82	0.04	2		492-494	3.54		1	
37-38	2.73		1		247-252	1.95	0.07	2		494-497	3.70		1	
48-49	2.81		1		254-256	2.61	0.04	2		497-500	3.42		1	
53.5-54.5	3.16		1		255-264	2.55		1		499-501	3.89	0.28	2	
56-59	3.41		1		266-271	2.37		1		502-504	2.92		1	
59-62	3.54	0.02	2	1	272-279	3.03		1		504-507	3.01		1	
67-69	3.49	0.08	3		286-291	2.82		1		510-513	3.02		1	
70-72	3.54		1		291-295	2.56		1		515-517	2.67		1	
73-75	3.14		1		295-298	2.49		1		517-519	2.63		1	
77-79	3.33		1		298-300	2.64		1		519-522	2.89	0.04	2	
84-87	3.50		1		300-303	2.40		1		523-525	2.68		1	
89-90	3.46		1		303-306	2.40		1		526-529	2.62	0.13	2	
93-95	2.92	0.04	2		307-309	2.34		1		529.5-532	3.18	0.08	2	
95-97	2.97	0.29	2		310-312	2.29		1		535-537	2.74	0.06	2	
100-103	2.71		1		312-316	2.54		1		537-539	4.53	0.38	2	
104-107	3.00		1		320-322	2.12		1		539-541	3.51	0.47	3	
109-111	2.73		1		322-324	2.17		1		541-544	3.08	0.16	3	2
114-116	2.56	0.22	3	1	325-328	2.18		1		544-547	3.53	0.06	2	
116.5-119	3.52	0.22	2		328-330	2.10		1		544-547	3.48		1	
119-122	2.53		1		330-332	2.25		1		547-550	3.57	0.31	3	
119-126	2.75		1		333-334	2.13		1		550-553	2.62	0.09	2	
123-126	2.52		1		334-336	2.09		1		553-556	2.31	0.16	2	
128-130	2.92		1		336-338	2.00		1		560-563	2.41	0.15	2	
130-133	2.90		1		339-341	2.09	0.01	2		563-565.5	2.82	0.49	4	
133-136	3.37		1	1	341-343	1.92		1		566-568.5	2.43	0.24	2	
136-138.5	2.57		1		343-345	1.86		1		568-571	2.47		1	
138.5-141	2.80		1	1	345-347	2.05	0.02	2		571-573.5	2.69	0.07	2	
141-143.5	2.53	0.01	2		347-348	1.95		1		573.5-575.5	2.55		1	
143.5-146	2.38	0.09	2		381-383	1.98		1		575.5-578.5	2.75	0.12	3	
146-148	2.40		1		384.5-7.5	2.25		1		578.5-581	2.55	0.11	2	
148-150.5	2.28		1		384.5-387.5	1.89		1		581-584	2.73	0.07	2	
150.5-153	2.39		1		384.5-387.5	2.05		1		584-587	3.27	0.06	2	
153-155	2.54	0.06	2		393-395	2.14		1		587-590	3.12	0.03	2	
155-157.5	2.93		1		395-397	2.40		1		590-593	3.36		1	
157.5-159	2.96	0.20	2		398-400	2.20		1		593-595	3.16		1	
159.5-162	3.17	0.32	2		400-402	2.29	0.19	2		596-598	2.98		1	
162-164.5	2.72	0.02	2		402-404	2.23	0.05	2	1	598-600	3.32	0.03	2	
164.5-167	2.54	0.12	2	1	404.5-407	3.04	0.29	4		600-603	3.34	0.27	2	
167-170	2.95	0.01	2		410-413	3.48		1		604-606	2.87	0.01	2	
170-172.5	2.61		1		413-415.5	2.17		1		607-610	2.35	0.04	2	
172.5-175	2.53	0.32	2		415.5-418	2.21	0.17	2		610-612.5	2.23	0.00	2	
177-179	2.15	0.03	2		418-420.5	2.35	0.09	2		613-616.5	2.26		1	
179.5-181.5	2.16		1		421-424	3.79	0.25	2		616.5-619.5	2.20		1	
185-187	2.34		1		423-426	2.45		1		620-623	2.04		1	
187-190	2.25		1		426-429	2.42	0.27	3	1	623-626	2.10	0.02	2	
190-193	2.29		1		429-432	2.31		1		627-630	1.86		1	
193-195	2.46		1		432-435	2.84	0.74	3		630-633	1.99		1	
195-198	2.47		1		435-438	2.75	0.25	2		633-636	1.92	0.18	2	
198-201	2.70		1		438-440	2.46	0.29	3		636-638	1.70		1	
201-203	2.74	0.04	2		440-442	2.37	0.11	3		638-640	2.04	0.29	2	
203-205	2.68		1		442-444	2.33		1		640-642	2.11	0.05	2	
207-209	2.70	0.16	2		444-446	2.24		1		642-644	2.01		1	
210-214	2.58	0.07	2		446-450	2.20		1		644-646	2.20		1	
214-217	2.80	0.20	2		456-458	2.35		1		646-648	2.00	0.10	2	
217-220	2.92	0.06	2		458-462	2.72		1		651-657	1.96		1	
220-222	2.93	0.38	2		464-466	3.23		1						

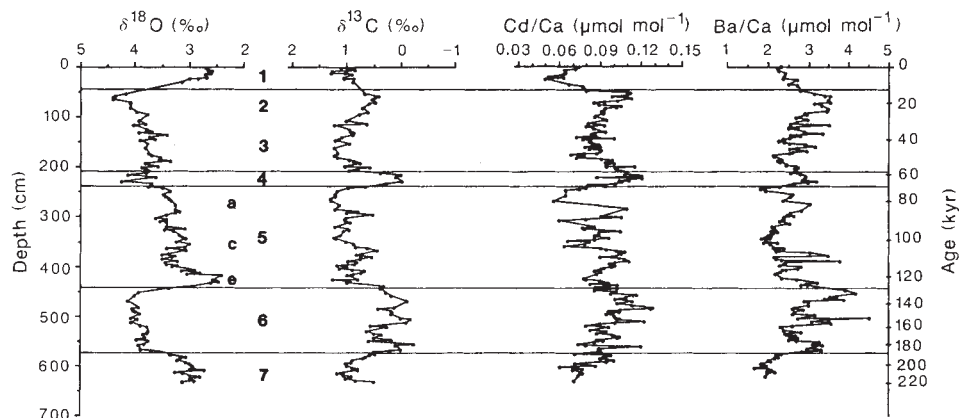
For depths with 2 replicates, standard deviations (SD) are differences from the mean. *n* is the number of replicates included in the mean and *r* is the number of replicates rejected from the mean.

* *C. kullenbergi*.

in the Ba content of the oceans over the past 200 kyr, however, because the interglacial minima are of similar magnitude between stages 1, 5 and 7. The question of change in oceanic Ba content cannot be answered adequately without a complementary Pacific record.

Two previous studies of core 4PC indicated that down-core variability in benthic foraminiferal Cd (and some of the down-core variability in $\delta^{13}\text{C}$) can be explained by variations in the flux of nutrient-depleted water to this site^{5,22}. Such variations would affect Ba in a similar manner because warm surface ocean

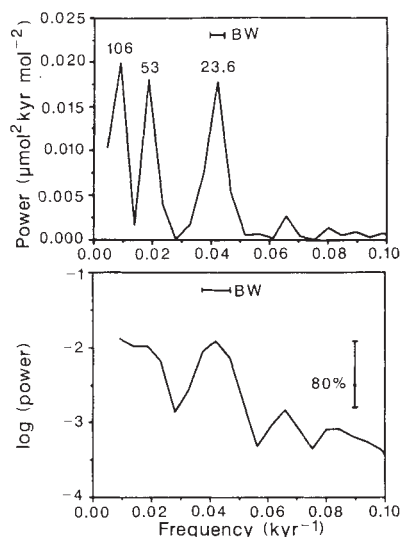
FIG. 1 Ba/Ca of benthic foraminifera (mostly *Uvigerina* spp.; see Table 1) plotted against depth for core 4PC. Also illustrated are oxygen isotope (*C. wuellerstorfi*), carbon isotope (*C. wuellerstorfi*) and Cd/Ca (mostly *Uvigerina* spp.) data for the same core⁵. Stage boundaries are based on the oxygen isotope record. Depths below 348 cm have been corrected by -32 cm because of a core void between 348 and 380 cm. The age model plotted on the right-hand side of the figure is based on correlation of the oxygen isotope record with a stacked oxygen isotope record¹⁷. ($\delta^{18}\text{O}(\text{‰}) = \{[(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}}] - 1\} \times 1,000$, where standard is PDB (NBS20); $\delta^{13}\text{C}$ is defined similarly, standard is PDB.)



waters are depleted in Ba. The fact that records of all three tracers indicate higher nutrients in Atlantic deep waters in glacial times (during which there is reduced flushing) suggests that at least some of the temporal variability in each tracer is the result of variations in the thermohaline circulation of the North Atlantic.

Spectral analysis has been applied to our Ba data and to the Cd, carbon and oxygen isotope data^{5,22} to evaluate the frequency response of these records with respect to the Earth's orbital parameters. The technique is based on the fast Fourier transform (FFT) method and is described in ref. 22.

Figure 2 illustrates periodogram and log power spectra from the benthic foraminiferal Ba record of core 4PC. The strongest power is found for frequencies of 0.0094 (106 kyr), 0.0189 (53 kyr) and 0.0425 (23.6 kyr). The power at 106 kyr is seen in almost all palaeoclimate records and corresponds to the period of eccentricity in the Earth's orbit. The power at 106 kyr in the Ba record accounts for ~22% of the total variance (area under the peak). The power at 23.6 kyr corresponds to the period of precession in the Earth's orbit. The peak at 23.6 kyr is the dominant peak for Ba, accounting for ~34% of the total variance in the spectra. The occurrence and magnitude of the peak at 53 kyr (24% of the total variance) is surprising²⁴⁻²⁶. Although this periodicity corresponds to the beat frequency between obliquity and precession, it is not a component frequency of insolation forcing. (A similar situation exists for the eccentricity modulation of precession and the 100-kyr climate cycle.)



The 53-kyr component of the Ba record is approximately in phase with the beat between obliquity and precession in the Northern Hemisphere. This observation needs to be confirmed by other long records before speculating about its cause.

The dominance of the 23-kyr precessional cycle in the Ba spectra stands out as a key factor that distinguishes the Ba record from that of Cd and carbon isotopes. Spectral analysis of August sea surface temperature in nearby core V30-97 indicates dominance of precessional power similar to that observed in the Ba spectra from core 4PC¹⁸. As Cd, Ba and carbon isotopes all potentially record variations in the flux of nutrient-depleted waters to the deep Atlantic, this difference suggests that either Ba is more sensitive than Cd or carbon in recording precessional forcing of deep-water formation or, that variability in these three tracers is forced by more than one mechanism. The first hypothesis seems less likely because Cd, which is more depleted in warm surface waters than Ba (that is, has a greater dynamic range), should always be more sensitive to variations in the flux of nutrient-depleted surface water. The second hypothesis is intriguing because it suggests that some of the temporal change in Ba might reflect a process that does not leave a prominent imprint on the records of Cd and carbon isotopes. This hypothesized process or mechanism must be strongly forced by the 23-kyr precessional cycle. A model developed in ref. 16 suggests that differences in the distribution of Ba and Cd (and carbon isotopes) in the oceans of the Last Glacial Maximum might be

FIG. 2 Periodogram and log power spectra for the core 4PC Ba record. Bandwidths (BW) and 80% confidence intervals are indicated. The power spectra are based on two-band averaging. See ref. 22 for details of spectral analysis. The record is first interpolated to create a record of 256 equally spaced points. To minimize spurious spectral properties, the interpolated record is detrended (subtraction of the linear trendline) and tapered (the first and last 10% of the record are multiplied by a function that varies from 0 at the ends of the records to 1 at the inside 10%, following the shape of a half-bell-shaped cosine); these changes reduce the resolution and amplitude of spectra peaks slightly. This modified record is then transformed using the discrete fast Fourier transform algorithm, which transforms the 256 real data points in time to a series of 128 real (a_j) and imaginary (b_j) coefficients as a function of frequency (where $f = 1/T, 2/T, \dots, 128/T$, where T is the record length). The periodogram is the power ($a_j^2 + b_j^2$) as a function of these frequencies. To improve the statistical properties of the periodogram, the power in adjacent frequency bands is averaged to provide a power spectrum. The 80% error bar indicates the amplitude of peaks exceeded only 20% of the time by a random noise signal. Although this method is different from the autocovariance technique also used to calculate spectra, the two methods are mathematically equivalent and no significant differences can exist between the methods when both are applied cautiously to avoid artefacts.

the result of the sensitivity of barium in the deep Atlantic to enhanced upwelling and the subsequently increased particulate Ba fluxes in the glacial Atlantic. There is some evidence to tie precessional forcing to enhanced equatorial upwelling in the Atlantic; when perihelion is aligned with boreal winter the zonal velocity of the trade winds is believed to be stronger and the equatorial Atlantic experiences maximum divergence and increased productivity^{27,28}. Presumably, particulate Ba flux out of the euphotic zone is greatest during these periods²⁹⁻³¹, which would reinforce a coincidence of Ba maxima with minimum

insolation during boreal summer (aphelion). A test of this hypothesis is to examine the phase relationship between faunal temperature indices for the equatorial Atlantic²⁸ and the Ba record. Such a comparison suggests that high barium in North Atlantic deep water lags sea surface temperatures of the cold equatorial Atlantic by up to 2–4 kyr. Although this lag is barely within the resolution of the data, it could be explained by vector addition of equatorial upwelling and deep-water formation (4-kyr lag relative to sea surface temperature of the equatorial Atlantic). □

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The role of hydrogen in the electrical conductivity of the upper mantle

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INTERPRETATIONS of the observed electrical conductivity of the upper mantle have relied on laboratory studies of dry olivine and basaltic melts^{1,2}. These studies indicate that the conductivity of the asthenosphere is higher than that of olivine, suggesting that partial melting or other high-conductivity mechanisms such as grain-boundary phases are required^{2,3}. Recent experimental studies^{4–6}, however, have demonstrated that various kinetic processes in silicate minerals are greatly enhanced by the dissolution of hydrogen. As hydrogen is probably present in the upper mantle, studies on dry olivine may not therefore be relevant to the real Earth. Here I use experimental data on the solubility and diffusivity of hydrogen in olivine^{5,7} to estimate its effect on the electrical conductivity. I conclude that the observed high conductivity in the asthenosphere can be attributed to solid-state conductivity in olivine if a small amount of hydrogen is present. Partial melting of the asthenosphere on a global scale is therefore not required.

The electrical conductivity of rocks depends on several factors including temperature, pressure, degree of partial melting and the chemical characteristics of the environment (such as oxygen fugacity)^{1,2,8}. Experimental studies on dry (water- or hydrogen-free) olivine suggest that implausibly high temperatures ($T > 1,500^\circ\text{C}$) are required in the asthenosphere to explain the high conductivity observed ($\sim 0.1\text{--}0.01\text{ S m}^{-1}$)^{1,2,9,10}, if solid-state conduction through bulk olivine crystals is the assumed mechanism. These observations have been interpreted as providing strong evidence for the existence of partial melt or of other mechanisms for high conductivity^{2,3}.

Recent experimental studies have demonstrated, however, that solid-state transport in silicates is made considerably more facile by the dissolution of a small amount of hydrogen^{4–7}. Infrared spectra of olivine annealed in a hydrous environment show absorption peaks due to the OH stretching vibration^{5–7,11,12}. These observations indicate that hydrogen (or water) is incorporated in olivine as a charged species, and so should increase the electrical conductivity if its solubility and mobility are large enough. Thus studies on dry olivine may not be relevant to the real Earth if some hydrogen or water is present in the upper mantle¹³. The electrical conductivity of olivine in the presence of hydrogen or water has been measured experimentally only at atmospheric pressure¹⁴; it is difficult to extrapolate these data as the solubility of hydrogen probably increases significantly with pressure. It is possible, however, to estimate theoretically the effect of hydrogen on conductivity once its concentration and mobility in olivine are known.

The contribution of charged, mobile species to the electrical conductivity of a solid is given by the Nernst–Einstein relation¹⁵

$$\sigma = fDcq^2/kT$$

where σ is the electrical conductivity, f is a numerical (correlation) factor approximately equal to unity, D is the diffusivity, c is the concentration, q is the electrical charge of the charged species, k is the Boltzmann constant and T is the temperature. The validity of the Nernst–Einstein relation for dry olivine has been demonstrated in refs 16 and 17, where it was suggested that electrical conduction in dry olivine at high temperatures ($T > 1,200^\circ\text{C}$) is due to diffusion of Mg^{2+} (or Fe^{2+}), although at lower temperatures the charge carriers are holes^{8,16}.

I have used the Nernst–Einstein relation to calculate the electrical conductivity in olivine containing hydrogen (Fig. 1). I assume that the hydrogen-related charge carriers have a charge $q = +e$ and I use the values of diffusivity determined experimentally⁷ at a pressure $P = 300\text{ MPa}$. Because of the uncertainties in the hydrogen content of the upper mantle, the conductivity was calculated for several values of this quantity. Experimental studies⁷ show that the solubility of hydrogen at $P = 300\text{ MPa}$ is

20–80 p.p.m. H/Si, and both theoretical and experimental studies suggest that the solubility of hydrogen increases with pressure^{5,6,19}. At asthenospheric pressures (~3–5 GPa), I therefore assumed values of 100 and 1,000 p.p.m. H/Si. The calculations indicate that the electrical conductivity of olivine increases significantly when hydrogen is present (Fig. 1). The conductivity at a realistic asthenospheric temperature of $T \approx 1,300^\circ\text{C}$ (ref. 18) is close to that estimated from geomagnetic sounding ($\sim 0.1\text{--}0.01\text{ S m}^{-1}$)^{9,10}.

Some simplifying assumptions have been made in this calculation. First, no correction was made for the pressure-dependence of hydrogen diffusivity, for which no experimental results are available. This assumption is probably justified in view of the observed small activation energy for hydrogen diffusion, which implies a small activation volume. Second, the solubility of hydrogen may depend on oxygen partial pressure (and oxide activity)^{7,19,20}. This effect is, however, relatively minor. Moreover, as the oxygen fugacity in the upper mantle is not well constrained, I have not considered its effect in detail. Third, the experimental data on hydrogen diffusivity were obtained at temperatures of 800–1,000 $^\circ\text{C}$, and extrapolation to higher temperatures was made assuming the same activation energy. This extrapolation probably gives a lower bound for the conductivity at higher temperatures, because activation energies often increase with increasing temperatures (Fig. 1).

The results in Fig. 1 were measured along the axis of highest conductivity. Because the hydrogen diffusivity is anisotropic⁷, the actual conductivity of olivine aggregates will be somewhat lower because of the spread in orientations.

I have assumed that the predominant charge carriers are protons. Another possibility is that the dissolution of hydrogen increases the concentration and/or mobility of other charged species (such as Mg^{2+} or holes). Karato *et al.*⁶ found that creep

controlled by Mg^{2+} (or Fe^{2+}) diffusion, and thus the diffusion of the ions themselves, is enhanced in the presence of hydrogen. But as the details of this effect are not known, I have not considered it here.

These uncertainties make it difficult to quantify the effect of hydrogen on the electrical conductivity of olivine; but the qualitative conclusion that the conductivity is enhanced seems sound. This conclusion could be tested readily by experiments at high confining pressures. By comparing calculated values of the conductivity with geophysical observations, one can obtain an estimate of the hydrogen content in olivine in the asthenosphere (Fig. 1). For a conductivity of $0.1\text{--}0.01\text{ S m}^{-1}$ at a temperature of $1,300^\circ\text{C}$, the hydrogen content is 200–2,000 p.p.m. H/Si. These values, however, depend on the absolute value of conductivity in the asthenosphere, which is somewhat uncertain because it depends on the (unknown) thickness of the high-conductivity layer^{9,10}. Also, these estimates assume that other mechanisms for high conductivity, such as the presence of grain-boundary phases, do not operate.

This estimate of hydrogen content is higher than that determined⁷ at $P = 300\text{ MPa}$ but is reasonable when one takes into account the enhancement expected at higher pressures. No detailed experimental studies of this pressure enhancement have been made, but comparison of creep experiments⁶ carried out for different water fugacities suggests that the solubility of water- or hydrogen-related defects increases with pressure as P' , with $r \approx 1/3\text{--}1/5$. This implies that the solubility at asthenospheric pressures (3–5 GPa) is higher than at 300 MPa by a factor of two to three, which is consistent with the range of solubilities estimated above.

Significant enhancement of the solid-state kinetics is possible for water (hydrogen) contents much lower than that required to reduce significantly the solidus temperature and thus change the chemical composition of the melt (present at $\sim 0.1\text{ wt\%}$ or higher)²¹. In other words, hydrogen could enhance the upper-mantle conductivity without causing extensive partial melting in much of the asthenosphere. Indeed, the latter seems not to occur: the chemical composition of mid-ocean-ridge basalt is consistent with the partial melting of 'dry' (in the petrological sense, that is, water content less than $\sim 0.1\text{ wt\%}$) peridotite²², implying that partial melting of the oceanic upper mantle is limited to the region near oceanic ridges. Given the results of recent experimental studies on anelasticity (ref. 23, and I. Jackson and M. S. Paterson, manuscript submitted, *Nature*), it seems that all of the geophysical anomalies in the asthenosphere can be attributed to solid-state processes. □

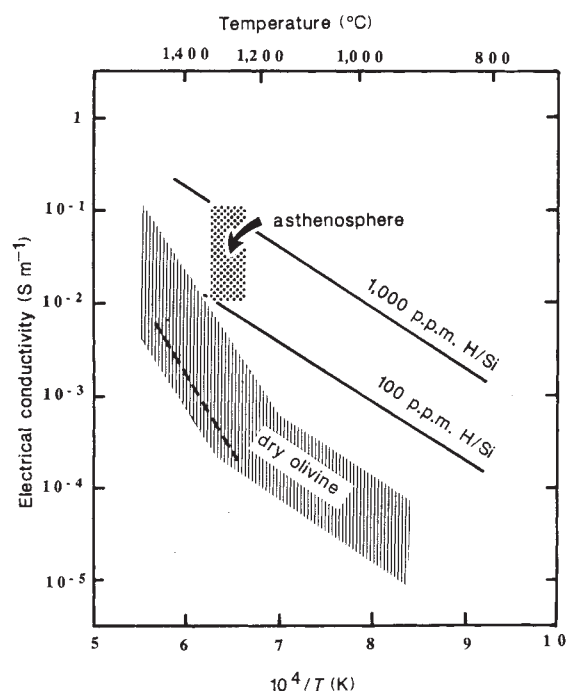


FIG. 1 Electrical conductivity in olivine as a function of temperature and hydrogen content. Data for dry olivine correspond to the hatched region^{1,8}. The dashed line shows the electrical conductivity calculated from the diffusion coefficients of Mg^{2+} (or Fe^{2+}) under dry conditions. Solid lines show the electrical conductivity calculated from experimental⁷ diffusivity of hydrogen. The dotted region indicates the range of conductivity estimated from geomagnetic soundings and the estimated temperature of the asthenosphere. This region coincides with the estimated conductivity for a hydrogen content of $\sim 200\text{--}2,000$ p.p.m. H/Si.

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Chaotic, subduction-like downflows in a spherical model of convection in the Earth's mantle

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NUMERICAL models of three-dimensional, nonlinear, thermal convection of very viscous fluids have recently been developed in rectangular^{1–4} and spherical^{5–12} geometries as analogues of convection in the Earth's mantle. Here we describe model calculations for a compressible fluid in a three-dimensional spherical shell with 80% of the surface heat flow generated within the model mantle, in agreement with estimates for the Earth's mantle¹³. The Rayleigh number and the numerical resolution for these calculations are greater than those of our previous studies^{9,10} of internally heated convection in a spherical shell. Our numerical solutions are strongly chaotic, with surface planforms dominated by long curvilinear downflows reminiscent of the descending slabs in the Earth's mantle. Although analogy to the Earth's mantle is necessarily imperfect, owing to, for example, the absence of variable viscosity and rheology—and hence of lithospheric plates—our results suggest that descending slabs play an important part in driving mantle convection, and that their chaotic evolution may influence the spatial and temporal behaviour of plates and thus the dispersal and aggregation of continents.

The numerical model⁷ solves the nonlinear equations of motion that describe thermal convection of a newtonian (linear viscosity), infinite Prandtl number (highly viscous), anelastic compressible (no sound waves) fluid in a three-dimensional spherical shell for conditions that approximate the Earth's mantle. The thermodynamic variables and the three components of velocity are expanded in spherical harmonics (up to degree and order 85) to resolve their horizontal structures and in Chebyshev polynomials (up to degree 30) to resolve their radial structures. The nonlinear advection and viscous heating terms are calculated each timestep in grid space on 256 Fourier longitudinal levels, 128 Legendre colatitudinal levels and 33 Cheby-

shev radial levels. Our numerical solution is adequately resolved because the kinetic and thermal energies drop several orders of magnitude as functions of the horizontal and radial wave numbers and because the solution is in good agreement with a solution at a lower numerical resolution (spherical harmonic degree and order up to 63).

Our model lacks several important features required of fully realistic numerical simulations of mantle convection in the Earth. One is a viscosity that depends on temperature, pressure and the state of stress. Another is a variable rheology simulating the behaviour of tectonic plates. Phase and chemical transitions in the mantle also need to be modelled. Although these features will certainly affect the spatial and temporal details of the convection, we first need to learn what our present model reveals about the basic properties of time-dependent three-dimensional compressible convection in a spherical shell.

We examine a case that is partly heated within the model mantle ($10^{-14} \text{ W kg}^{-1}$) and partly heated from the underlying core. An adiabatic polytrope with an index of 0.4 and a Grüneisen parameter of 1.1 determines the profiles of the reference-state pressure, density and temperature that approximate the Earth's mantle and about which the thermodynamic variables are perturbed. Impermeable stress-free boundary conditions are imposed on the velocity. The model temperatures are set to 3,270 K at the inner (core/mantle) boundary and 1,070 K at the outer boundary. Of the total temperature drop across the model mantle, 1,200 K is due to adiabatic compression and the rest, 1,000 K, is the superadiabatic temperature drop, which drives the convection. We specify a constant dynamic viscosity of $5.6 \times 10^{22} \text{ kg m}^{-1} \text{ s}^{-1}$ and a constant thermal conductivity of $23 \text{ W m}^{-1} \text{ K}^{-1}$. Owing to computing limitations, the thermal conductivity adopted in the model is about five times larger than the conductivity of actual mantle rocks. The volume-averaged Rayleigh number Ra (a measure of the convective vigour⁷) due to both the superadiabatic temperature drop and the internal heating is 1.6×10^6 . This is ~ 200 times greater than the critical Rayleigh number required for the onset of convection, but probably still ~ 10 times smaller than the average value for the Earth's mantle. We use a timestep of 10^6 years, one quarter of the Courant condition, and start from small random initial perturbations in the entropy (temperature) field⁷.

The resulting time-averaged heat flows through the inner and outer boundaries are $1 \times 10^{13} \text{ W}$ and $5 \times 10^{13} \text{ W}$, respectively. Thus, $\sim 80\%$ of the heat conducted through the outer boundary is generated in the model mantle. The total heat flow through the outer boundary is comparable to the heat flow through the Earth's surface. In the bulk of the model mantle $>90\%$ of the heat flow is convective and $<10\%$ is conductive. Maximum convective velocities in the model are $\sim 30 \text{ mm yr}^{-1}$, comparable to the observed velocities of tectonic plates.

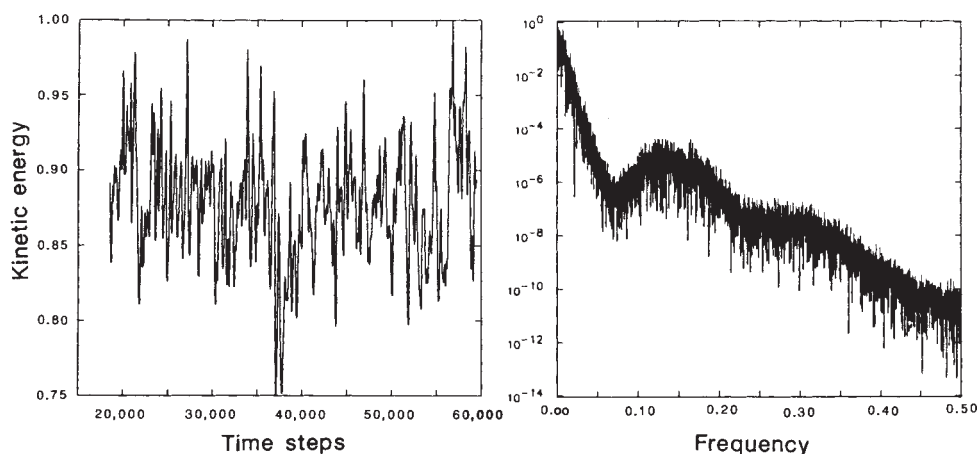


FIG. 1 Total kinetic energy as a function of the timestep and its power spectrum as a function of frequency (in cycles per second scaled by the timestep). Both plots are normalized to unity. For this long-time integration we truncated the spherical harmonic expansions at $l=63$ instead of 85.

The total kinetic energy in the model mantle is plotted in Fig. 1 as a function of time for the last 40,000 timesteps of the calculation. The large amplitude variation illustrates the time dependence of the solution; however, the basic qualitative features of the convection do not change over this period of time. Also plotted in Fig. 1 is the square of the Fourier transform of the kinetic energy time series as a function of frequency. The broadband distribution of power is indicative of a chaotic system. In addition, the phase space representation of kinetic energy as a function of its time rate of change (not displayed here) shows no limit cycle or torus, indicating chaos. Two popular methods for calculating the fractal dimension^{14,15} failed to converge, suggesting a high fractal dimension for our three-dimensional convection. Our results support the conclusion, made on the basis of two-dimensional calculations¹⁶⁻¹⁸, that mantle convection in the Earth is chaotic. Our results suggest, however, that mantle convection is much more chaotic than the low-fractal-dimension solutions obtained with the two-dimensional models using low Rayleigh numbers and no internal or viscous heating.

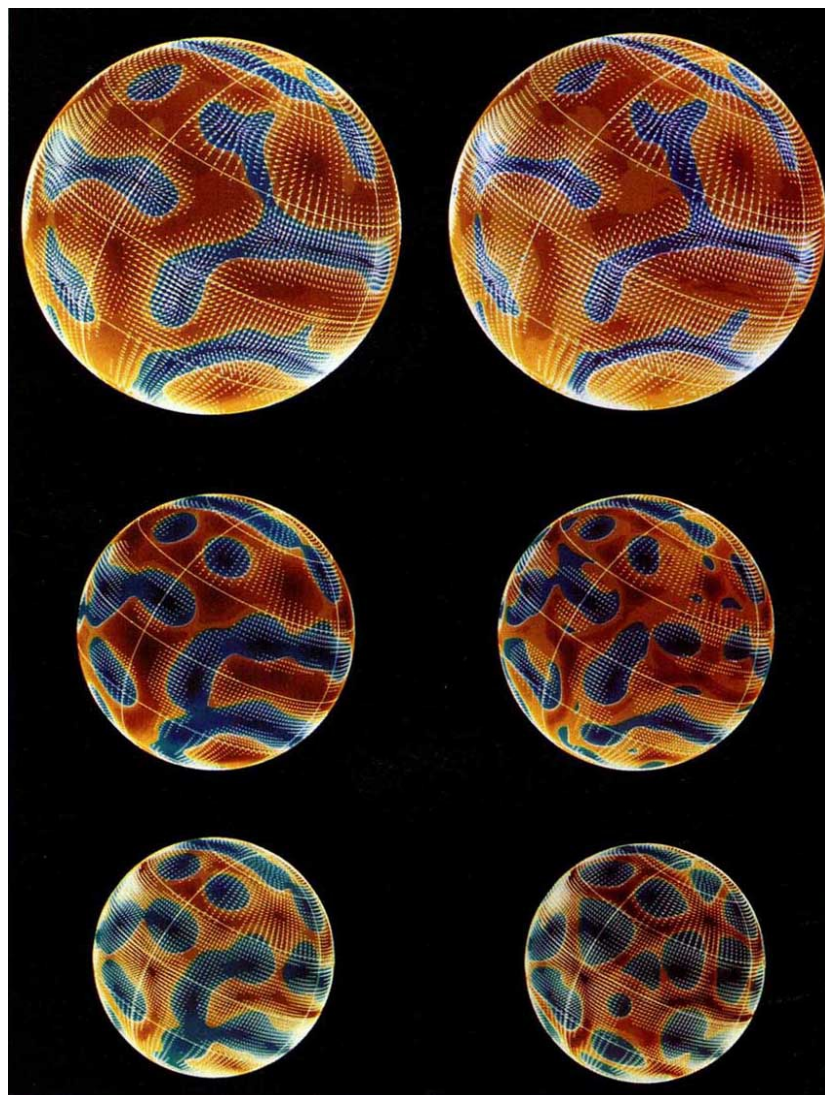
A typical snapshot of the spatial patterns of the temperature and velocity fields on three different surfaces of constant radius is shown in Fig. 2. In the upper part of the sphere, cold dense fluid tends to converge and sink in long narrow sheets surrounded by a weak background of warm buoyant diverging upflow. This thin sheet-like downflow, initiated by the thin thermal boundary layer at the top, tends to break up with

increasing depth into cylindrical downflow plumes that squeeze the hot fluid between them as they impinge on the inner boundary forcing hot cylindrical upflow plumes at the vertices of the connected network of warm fluid. These upflow plumes expand in the upper part of the sphere forming the weak background of upwelling there.

The formation of the upwelling plumes at the intersections of warm linear thermals is similar to the spoke pattern of convection observed in laboratory and numerical studies of plane-layer convection with constant viscosity and all heating from the bottom of the fluid layer^{2,4,19}. Purely bottom-heated convection in spherical shells does not result in such spoke patterns and in fact maintains upwelling plumes surrounded by downwelling sheets over the entire depth of the shell; the contrast with plane-layer convection is primarily because of the breaking of the mid-plane symmetry by the spherical geometry^{7,9,20}. Thus, the similarity of our internally heated solutions to bottom-heated plane-layer convection implies that the mid-plane symmetry is partially restored by internal heating in the spherical shell (by equalizing the temperature drops across the top and bottom boundary layers, for example). Unlike plane-layer convection, however, the network of linear thermals is more prominent in the temperature field (which is what the shadowgraphs in laboratory experiments detect), whereas the strong upwelling motion is primarily confined to cylindrical plumes.

Three snapshots, 200 million years apart, of the spatial patterns of the temperature and velocity fields in a typical cross-

FIG. 2 Plots (all at the same timestep) of convective velocities and temperatures in three different constant-radius surfaces (5,940, 4,350 and 3,770 km). These spherical surfaces are scaled according to their radii. The colours in the three plots on the left represent the radial component of velocity with a contour increment of 1.5 mm yr^{-1} . Reds and yellows represent upflow (a maximum of 12.0 mm yr^{-1}); blues represent downflow (a maximum of 19.5 mm yr^{-1}). The colours in the three plots on the right represent the temperature relative to the spherically averaged value at each radius with a contour increment of 50 K. Reds and yellows represent hot fluid (a maximum of +400 K); blues represent cold fluid (a minimum of -650 K). The arrows represent the direction and amplitude of the horizontal velocity in these surfaces. All are scaled in the same way, with a maximum of 20 mm yr^{-1} . Velocities less than 2 mm yr^{-1} are not plotted.



sectional slice through the spherical shell are illustrated in Fig. 3. A typical cold boundary layer instability at '12 o'clock' is developing in the first (top) plot. It has sunk to mid-depth in the second (middle) plot, and is spreading along the lower boundary in the third (bottom) plot. Figures 2 and 3 show how the spatial scale of the temperature is smaller than that of the velocity (owing to the small thermal diffusivity compared to

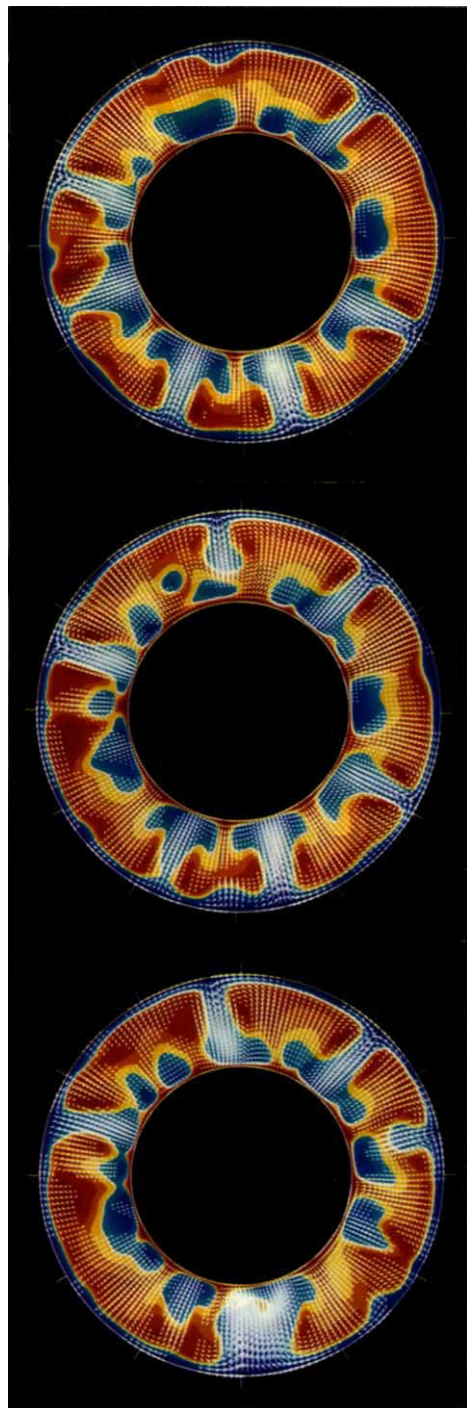


FIG. 3 Plots (in the same cross-sectional slice through the three-dimensional shell) of convective velocities and temperatures at three different times separated by 200 timesteps (200 million years) beginning with the top plot. The colours represent the temperature relative to the radially dependent adiabatic temperature profile with a contour increment of 50 K. Reds and yellows represent hot fluid (a maximum of 300 K); blues represent cold fluid (a minimum of -750 K). The arrows represent the velocities in this cross-sectional surface and are scaled the same way as those in Fig. 2.

kinematic viscosity) and how at certain times and places relatively hot fluid is dragged downward.

A movie of this numerical simulation shows the convection pattern continuously changing, with the hot upflow passively reacting to the dominant cold downflow. The long downflow sheets in the upper part of the sphere sometimes contract or break up, forming cylindrical-like downwelling features and at other times the downflow sheets link up forming even longer sheet-like features. Downwellings form spontaneously over regions that are warm at depth. Although the convection pattern is very chaotic, the basic spatial and temporal characteristics of the convection do not change after the decay of the initial transients.

We can compare these results to our previous calculations with 50% and 80% internal heating^{9,10} which were run at lower Rayleigh numbers and at a resolution a factor of two lower in both latitude and longitude. Although the previous patterns were time-dependent, the present results are more temporally complex and definitely chaotic. In our earlier calculations convection patterns tended to be anchored to the upwelling plumes which were relatively steady in number and location. In contrast, the upwelling plumes in our present calculations are more variable in number and location owing to their interaction with chaotic downwelling. At even higher Rayleigh numbers than in the present calculation (stronger thermal driving relative to viscous and thermal diffusion), convection will probably have greater spatial and temporal structure and modelling will require still better numerical resolution. Higher numerical resolution (and therefore more computing resources) will also be required when three-dimensional models account for variable viscosity, tectonic plates and phase and chemical transitions. It is difficult to predict how these needed improvements will alter the structure of the simulated convection. It is, however, encouraging that the sheet-like downwellings seen in our previous calculations at 80% internal heating¹⁰ also dominate the structure of convection here. This qualitative agreement gives us confidence that this downwelling pattern will continue to be an important feature as models of mantle convection become more realistic.

If the numerical model presented here is sufficiently representative of the Earth's mantle, the numerical results have significant implications for the nature of mantle convection. Convection in the model is predominantly controlled by the largely sheet-like downwellings, suggesting that the descending slabs in the Earth are the main drive for plate motions and mantle convection²¹. The prominent role of downwellings in convecting systems heated strongly from within has also been demonstrated in other numerical studies^{2,4,8-10} and in laboratory experiments^{22,23}. The lengthening of downwellings in the model along their axes suggests the possibility that subduction zones on Earth may evolve by propagation along their strike⁴. In our model, the downwelling sheets also drift perpendicular to their axes, similar to the lateral migration (perpendicular to strike) of descending slabs through the mantle²⁴. Because subducting slabs probably drive plate tectonics, their chaotic migration and propagation (inferred from these numerical calculations) may also have a role in the apparently random dispersal and aggregation of continents.

As the upflow in our calculations takes the form of cylindrical plumes, our results support the evidence that mid-ocean ridges are the result of passive rifting^{9,25}. The upwelling plumes in our model broaden with height. A temperature-dependent viscosity would probably focus the thermal and velocity anomalies of plumes into smaller-diameter cylindrical structures and flatten the heads of plumes more dramatically as they encounter the base of the strong lithosphere^{26,27}. These plumes probably weaken the lithosphere and determine where the lithosphere tears and spreads apart in response to slab pull. This may be one reason why many hot spots are preferentially located near mid-ocean ridges²⁸. □

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Enhancement of nitrogen deposition to forest trees exposed to SO₂

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DEPOSITION of atmospheric nitrogen compounds, particularly ammonia, is postulated to have a variety of deleterious effects¹, including damage to soils, trees and other vegetation^{1–6}. We report here that fumigation of coniferous trees in the open air with sulphur dioxide (13 and 22 p.p.b.—parts in 10⁹ by volume) produced an increased deposition of atmospheric NH₃ to foliage. A mechanism for co-deposition of NH₃ and SO₂ to wet surfaces has been suggested^{7,8}. As concentrations of NH₃ at the site are not unusually high (annual mean, 4 p.p.b.), this mechanism may enhance nitrogen deposition over large areas of Europe. It may also account for some results of experimental fumigations previously attributed to SO₂ alone.

The experimental site is located at Liphook, Hampshire, UK and uses a technique previously developed to fumigate cereals with SO₂ (ref. 10). In 1985 seven plots (Fig. 1) were planted with seedlings of Scots pine (*Pinus sylvestris*), Sitka spruce (*Picea sitchensis*) and Norway spruce (*Picea abies*)¹¹. Concentrations for SO₂ treatments were multiples (1.5 and 3 times) of sequential hourly mean concentrations measured in 1979–1980 at a rural site in central England¹². Target values for ozone treatments were a constant multiple (1.5 times) of the measured ambient O₃ at the site. Including ambient concentrations, this gives a 2×3 factorial design with one additional ambient plot (Fig. 1). Exposure to SO₂ commenced in May 1987 and O₃ fumigation occurred from March–December 1988 and May–December 1989.

Each October, eight trees of each species per plot were harvested for biometric and chemical analysis. The foliar content of nitrogen, sulphur and cations was determined by standard methods¹³. In summer 1989, throughfall collectors (diameter, 0.9 m) were fitted to four trees each of Scots pine and Sitka spruce in plots 2–7. Samples were collected fortnightly and analysed separately.

In October 1988 the foliar nitrogen content of Norway spruce showed a clear trend with SO₂ treatment, being 1.7 times higher in the high-SO₂ plots than in the ambient plots (Fig. 2). Sulphur content of the needles also reflected the SO₂ treatments (Fig. 2). A similar trend in foliar nitrogen was observed in Sitka spruce, but not in Scots pine. Needles of Norway spruce on plots without SO₂ fumigation were below the deficiency level of 12 mg N g⁻¹ in October 1988 (Fig. 2); needles of Sitka spruce on all plots were likewise deficient, whereas those of Scots pine remained above the deficiency level of 11 mg N g⁻¹ (ref. 14). Estimates of the total nitrogen content of needle biomass in October 1988 revealed that the Norway spruce and Sitka spruce contained more nitrogen (7.8 kg ha⁻¹ and 12.8 kg ha⁻¹, respectively) in the 22 p.p.b. SO₂ plots than trees in ambient plots.

There are several mechanisms that might explain this difference in nitrogen, including N₂ fixation, litter decomposition, reallocation from roots, changes in understorey vegetation and atmospheric inputs. Studies of the soil nitrogen cycle¹⁵ found no evidence for differences in N₂ fixation or nitrogen mineralization. It is possible that SO₂ effects have suppressed the nitrogen demand of understorey vegetation, but there is no evidence for this. Likewise, studies of below-ground biomass have shown no indication of treatment effects (although with large errors), and reallocation of nitrogen from needles prematurely lost in SO₂-treated plots would not supply enough nitrogen to explain the effect. The most probable explanation for the increased nitrogen appears to be a known mechanism for SO₂ enhancement of NH₃ deposition to wet surfaces^{7,8}. The solubility of SO₂ or NH₃ alone is limited by the pH of the acidic or basic solution they form, respectively, whereas with both gases together, the pH can remain at non-limiting values, so enhancing solubility and deposition. Evidence to support this comes from measurements of ammonium ion in throughfall and measured deposition to water surfaces within the plots.

Ammonium in throughfall has been measured from July 1989

FIG. 1 Map of Europe showing the location of the study site, the layout of the experimental plots and gaseous treatments. The plots were planted with 196 seedlings of each species at 1-m spacing in a 25-m diameter area within a 50-m diameter circle of gas sources¹¹.

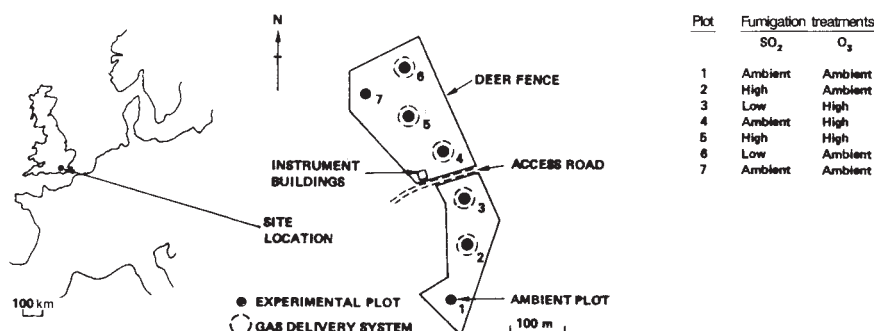


TABLE 1 Chemical composition of rain and throughfall (including stemflow) below Scots pine and Sitka spruce in open-air fumigation plots from 12 July 1989 to 24 January 1990

Species	Plot	Treatment concentration (p.p.b.)		Throughfall concentration ($\mu\text{eq l}^{-1}$)				
		SO ₂	O ₃	NH ₄ ⁺	NO ₃ ⁻	SO ₄ ²⁻	H ⁺	pH
<i>Pinus sylvestris</i>	2	21	19	192 (60)	115 (24)	1,016 (146)	276 (30)	3.6
	3	13	24	118 (25)	150 (39)	693 (86)	174 (24)	3.8
	4	6	24	93 (16)	169 (41)	535 (82)	100 (19)	4.0
	5	21	24	185 (37)	215 (39)	1,294 (136)	333 (38)	3.5
	6	12	19	86 (14)	119 (25)	720 (87)	185 (19)	3.7
	7	5	19	101 (23)	146 (35)	353 (47)	73 (15)	4.1
<i>Picea sitchensis</i>	2	21	19	102 (18)	173 (31)	1,015 (125)	289 (34)	3.5
	3	13	24	73 (11)	99 (20)	704 (78)	174 (23)	3.8
	4	6	24	93 (14)	97 (22)	445 (61)	39 (9)	4.4
	5	21	24	139 (23)	149 (26)	1,056 (97)	282 (32)	3.6
	6	12	19	95 (19)	107 (21)	784 (88)	172 (23)	3.8
	7	5	19	69 (14)	110 (24)	447 (92)	50 (10)	4.3
Rainfall, north site		5	19	51 (10)	33 (9)	90 (10)	19 (7)	4.7
Rainfall, south site		5	19	49 (15)	71 (22)	114 (17)	31 (18)	4.5

NH₄⁺ was determined by the indophenol blue colorimetric method¹³, and NO₃⁻ and SO₄²⁻ by ion chromatography. Values are the means from four replicate throughfall collectors sampled on 10 occasions (at fortnightly intervals unless there was no rainfall), with standard errors in brackets; pH values are calculated from the mean H⁺ concentration. Gaseous concentrations are the mean values over the period of throughfall sampling. Mean concentrations of ambient NO and NO₂ were 5 and 8 p.p.b. respectively. Ozonized air was water-scrubbed to remove contaminant N₂O₅ (ref. 11) during this period and the preceding fumigation in 1989.

TABLE 2 Chemical composition of water samples exposed in open-air fumigation plots during March 1990

Period	Plot	Treatment concentration (p.p.b.)		Concentration ($\mu\text{eq l}^{-1}$)				
		SO ₂	O ₃	NH ₄ ⁺	NO ₃ ⁻	SO ₄ ²⁻	H ⁺	pH
2-7 March (125 h)	1	3	25	53.8 (5.7)	3.9 (0.3)	20.1 (0.5)	0.5 (0.1)	6.3
	2	35	26	76.6 (5.7)	5.3 (0.3)	101.7 (2.5)	128.1 (0.8)	3.9
	5	38	34	78.6 (5.6)	5.4 (0.2)	109.8 (4.3)	145.7 (6.9)	3.8
	7	3	26	53.3 (0.8)	4.3 (0.2)	19.2 (0.5)	0.5 (0.1)	6.3
7-13 March (139 h)	1	3	21	83.7 (4.9)	14.5 (0.4)	48.7 (2.3)	16.7 (0.7)	4.8
	2	33	22	113.9 (5.2)	18.0 (0.3)	112.1 (6.7)	97.0 (8.3)	4.0
	5	35	32	112.7 (2.1)	17.5 (0.9)	144.1 (6.7)	151.3 (13.4)	3.8
	7	3	22	82.7 (4.0)	13.4 (0.7)	61.5 (1.1)	36.8 (2.7)	4.4

Deionized water (25 ml) was exposed in open polystyrene Petri dishes (8.7 cm diameter) at 1.5 m height and protected from rainfall in the ambient and high-SO₂ plots. Water levels were maintained daily. NH₄⁺, NO₃⁻ and SO₄²⁻ concentrations are mean values (with standard errors) for four replicate dishes, two each on the north and south edges of the trees, 12.5 m from the gas outlets (Fig. 1), compared with water in two sealed control dishes. Analytical methods are given in Table 1; pH values are calculated from the mean H⁺ concentration. Dilute sulphuric acid (25 ml; 0.17 mg l⁻¹ SO₄²⁻) kept in closed Petri dishes for 6 days showed no increase in NH₄⁺ concentration. NH₄⁺ analysis by the indophenol blue colorimetric method¹³ may also detect volatile amines²² and results were confirmed using ion chromatography. Mean concentration of ambient NO was <2 p.p.b. during each period and NO₂ was 3 p.p.b. from 3-7 March and 5 p.p.b. from 7-13 March. There is no evidence for NO₃⁻ deposition from N₂O₅ contamination of the ozonized air¹¹, which was water-scrubbed before fumigation during these periods.

and shows concentrations in high-SO₂ plots 1.9-times higher for Scots pine and 1.7-times higher for Sitka spruce than in the ambient plot (Table 1). Some of this NH₄⁺ may have been leached from internal sources, but it seems likely that a large fraction results from wash-off of dry deposited NH₃ following co-deposition with SO₂ (ref. 6). These data therefore suggest that dry deposition of NH₃ is enhanced in SO₂ fumigated plots. The NH₄⁺ in throughfall represents the excess of deposition and leaching over uptake, and this excess may have been insufficient to enhance the throughfall concentrations in the low-SO₂ treatment plots.

The deposition of NH₃ was evaluated by exposing dishes of deionized water for several days in the ambient and high-SO₂ plots. Analysis for NH₄⁺ (Table 2) suggests an additional deposition of 27 and 31 ng N cm⁻² d⁻¹ to the water surfaces in the high-SO₂ plots compared with those in the ambient SO₂ plots during the two periods. The much smaller increase in NO₃⁻ may

result from reactions between SO₂ and NO₂ in solution to enhance NO₂ deposition¹⁶. These data imply that SO₂ may enhance NH₃ deposition to all ecosystem surfaces, including leaf surfaces and mesophyll cells, bark and soils¹⁷⁻¹⁹. Calculated nitrogen deposition rates of ~10-20 kg ha⁻¹ yr⁻¹ are obtained using a deposition velocity of NH₃ to vegetation of 5-10 cm s⁻¹ (ref. 20), assuming a concentration of 1 p.p.b. NH₃ (the lower end of the range of annual mean concentrations reported for the UK⁹). Although throughfall composition (Table 1) and deposition to water surfaces (Table 2) strongly suggest that NH₃ is the source of the additional foliar nitrogen, some NH₃ may originate from the vegetation²¹, and then be captured by the foliage²².

Enhanced nitrogen deposition of this magnitude has important implications because 10-20 kg ha⁻¹ N has been proposed as a 'critical load' that should not be exceeded if damage to forest ecosystems is to be avoided²³. The potential for enhanced

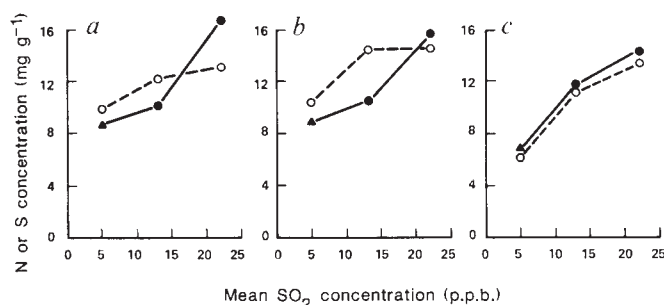


FIG. 2 Foliar concentrations of nitrogen and sulphur in Norway spruce trees exposed to SO_2 and O_3 by open-air fumigation and sampled in October 1988 (a, N in 1987 needles; b, N in 1988 needles; c, S in 1988 needles). Values are means of 8 trees for the 5 fumigated plots (○, ●), and 16 trees for the 2 unfumigated ambient plots (▲). Open symbols represent plots receiving ozone fumigation (30 p.p.b.) and filled symbols indicate ambient O_3 plots (24 p.p.b.). Exposure concentrations are annual means for October 1987 to September 1988. Ozone was generated from electric discharge in dry air forming contaminant N_2O_5 (ref. 11) that was removed (in 1989 only) by recirculating water in a packed-bed scrubber. Nitrogen content of O_3 -fumigated needles may be affected by N_2O_5 deposition before 1989, and by O_3 oxidation of SO_3^{2-} which may enhance NH_3 co-deposition with SO_2 (refs 7, 8) at pH values of surface moisture above 4.7. Nitrogen content was assessed after digestion with H_2O_2 - H_2SO_4 using the indophenol blue colorimetric determination of NH_4^+ (ref. 13). Sulphur content was determined after ashing with HNO_3 at 550 °C, using inductively coupled plasma emission spectrometry.

NH_3 deposition may be greater for mature forest trees, for which the mean leaf area index (LAI) is 3–5 (ref. 24), as compared with the trees in this experiment, for which the LAI is 1–2.5. Some results of experimental SO_2 fumigation or ambient air filtration may need to be reassessed, depending on whether charcoal filters were used, which remove half of the NH_3 (ref. 25), or materials containing KMnO_4 , which may remove it all. In addition, the use of nitrogen-sufficient plants may obscure the effect of increased atmospheric nitrogen supply on foliar nitrogen concentration²⁶. The nitrogen-sufficient Scots pine in this study did not show the same response as the spruces. Enhanced NH_3 deposition could also explain the increased foliar nitrogen observed during open-air SO_2 fumigation of cereals^{10,27}, increased performance of insects²⁷, mildew²⁸, and increased activity of glutamate dehydrogenase²⁹, all previously reported as solely SO_2 effects. The importance of NH_3 and NH_4^+ deposition in areas of high- NH_3 emissions is well known^{1–6}, but these data indicate that it may be important over wide areas where relatively low concentrations of SO_2 and NH_3 co-exist. □

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Acceleration of senescence in the collared flycatcher *Ficedula albicollis* by reproductive costs

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SENESCENCE is the innate deterioration of an organism that leads to a decline in its fitness components such as fecundity and survival expectations with advancing age^{1,2}. Williams³ proposed that the evolution of senescence could be understood in terms of age-specific pleiotropic gene action, where a mutation increases fitness early in life at the expense of reducing it later on. The basic idea, first proposed by Medawar¹, is that mutations acting early in life are more sensitive to selection than those acting later, as the early effects would be expressed in more individuals than would the late effects. A cost of reproduction, where an increase in reproductive rate reduces future survival or fecundity, could be the kind of pleiotropic gene action that generates senescence^{2,3}. Evidence exists for such costs in animals and plants^{4–8}, but their role in the evolution of senescence is so far not clear². Most empirical work has been with fruit-flies in laboratory studies, where selection experiments indicate negative genetic correlations between early and late fitness components^{9–12}. But it is not certain how applicable the results of such studies are to natural populations. Here we report that the clutch size of older individuals in the collared flycatcher *Ficedula albicollis* is affected by their reproductive effort early in life. This provides empirical support for Williams's idea that costly reproduction accelerates senescence for fertility in a vertebrate species under natural conditions.

Time, energy and nutrients are often limited for individuals and have to be allocated between somatic maintenance, growth and reproduction. General life-history theory^{4,13,14} predicts that a high reproductive effort early on in life should be costly for an individual and lead to reduced lifespan or subsequent fertility. There have been two approaches to the investigation of these costs: genetic studies^{3,4,15}, and phenotypic studies using environmental manipulations of reproductive rate^{4,16–17}. Here we report the use of the second approach.

We have studied the population biology of the collared flycatcher on the southern part of the island of Gotland^{18,19} in the Baltic from 1980 to the present. The collared flycatcher *Ficedula albicollis* on the island of Gotland forms an isolated population so that survival and reproduction of both parents and their offspring can be assessed throughout their entire lifespan^{18,20}.

The reproductive performance of collared flycatcher females increased during early life and decreased at older ages, although there was no significant change in survival probability (Fig. 1). A similar pattern has also been observed for many other bird species²¹. Because we have found no evidence of a higher frequency of external injuries or defects in these old birds, the

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TABLE 1 Effects of age of first breeding and current age on clutch size in female collared flycatchers

	Cross-section		Longitudinal	
<i>n</i>	516		165	
Source	<i>F</i> -value	<i>P</i>	<i>F</i> -value	<i>P</i>
Age (current)	1.27	0.282	1.93	0.149
Age (first breeding)	6.19	0.013	8.96	0.003
Interaction	0.88	0.414	0.11	0.894

Two-way factorial ANOVA; mean values, standard errors and sample sizes are shown in Fig. 2. We regarded individuals that were not captured by us during their first year of life as nonbreeders. This category probably included individuals that actually bred during their first year, but because breeding dispersal is very low¹⁹, only very few of our putative nonbreeders are likely to have bred outside the study area. Although this category might include a few birds that did have some reproductive effort in their first year, our comparison for differences is conservative.

decrease in reproductive performance is probably caused by some innate deterioration of the individual. Thus, collared flycatcher females older than 3 years clearly show signs of senescence for fertility.

By what processes could a seemingly maladaptive character like senescence for fertility evolve? A cost of reproduction could be a candidate for the gene action proposed by Williams^{2,3}. To

TABLE 2 Effects of age and brood size manipulations on clutch size in female collared flycatchers in the three years after the experiment

	All birds				Only one-year-old			
	Cross-section		Longitudinal		Cross-section		Longitudinal	
<i>n</i>	201		74		112		50	
Source:	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Age	0.85	0.43	1.84	0.166	0.19	0.83	0.31	0.73
Treatment	8.15	0.005	19.1	0.0005	4.27	0.041	4.87	0.002
Interaction	2.97	0.054	0.23	0.79	1.76	0.18	0.16	0.85

Two-way factorial ANOVA analyses for all birds irrespective of age (age did not differ between groups (U-test, $P > 0.5$) and for birds that were one year old during the manipulation are presented. Mean values, standard errors and sample sizes are shown in Fig. 2b,c. We used individuals from a previously published brood size experiment⁶ that had either increased or reduced broods (one or two young). Because these manipulations were found to affect both subsequent clutch size⁶ and fledgling success (L.G., unpublished results), the number of individuals of older ages that could be used was reduced because many were manipulated in several years. Because the number of individuals is small in the older age classes we could only use individuals up to the age of 4 years (that is, 3 years after the experiment). A one-way ANOVA showed a significant difference between enlarged broods and both control and reduced broods ($P < 0.05$, Scheffe's test) in years after the experiment, whereas there were no significant difference between reduced and control broods. These results are probably because reduced broods have heavier young²² with higher survival⁶, thereby reducing any difference in effort between reduced and control females. We therefore pooled the data of control and reduced broods.

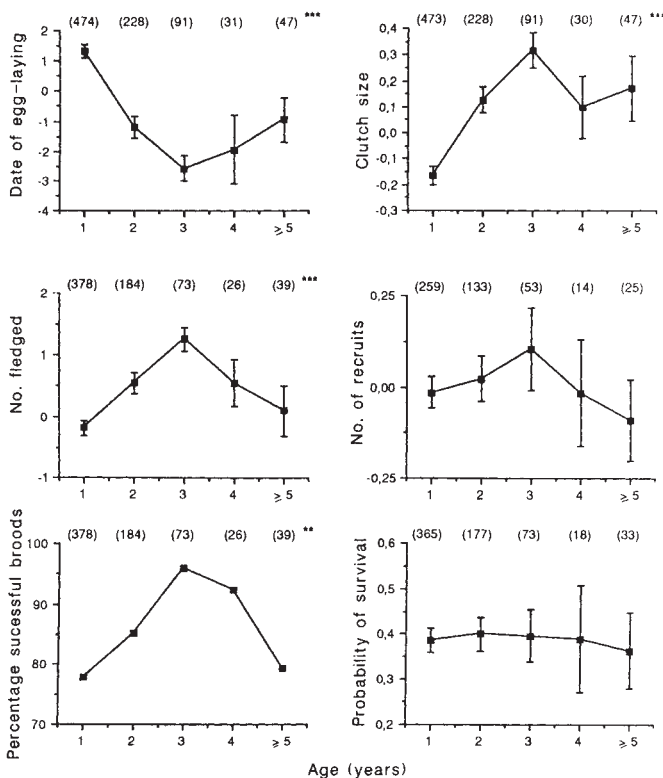


FIG. 1 Cross-sectional data of age and reproductive performance in collared flycatcher females breeding between 1980 and 1987. All data, except percentage of successful broods and probability of survival were standardized (mean = 0) for each year. Sample sizes are shown in brackets and *P* values are indicated as ** < 0.01, *** < 0.001. The test used was one-way ANOVA, except for percentage successful broods and survival rates, where a G-test was used. In the age-class of ≥ 5 -year-old females we included birds with an unknown exact age at first trapping and therefore the sample size was larger than for 4-year-old females. The shown parameters are added effects of age, but, also when creating independent parameters by correcting for date of egg laying (residual analysis), clutch size and number of fledged young per laid egg showed the same relationship to age ($P < 0.01$), but number of recruits per fledged young was not significantly related to age.

test Williams's idea that reproductive effort early in life affects fecundity late in life we used two methods. First, we compared birds breeding for the first time at the age of 1 year to those that started to breed at 2 years of age. Second, we compared individuals with experimentally enlarged brood size to those with reduced or unmanipulated brood size. The first approach is purely correlative, and therefore less powerful than the second⁴⁻⁶.

Collared flycatcher females that did not breed during their first year laid larger clutches throughout their subsequent life than did females starting to breed in their first year (Table 1; Fig. 2a). It is not likely that 'first-' and 'second-year' breeders represent two different strategies (that is, genetic lines with late breeding and high fecundity versus early breeding and low fecundity) as the number of breeding seasons is the most important factor affecting lifetime reproductive success (LRS) in the collared flycatcher¹⁸, thereby promoting a strong selection for breeding in the first year of life¹⁸. Furthermore, a comparison of birds used in this analysis showed that first-year breeders had higher LRS (1.24 ± 0.08 recruits) than second-year breeders (0.90 ± 0.14 recruits; $z = -2.03$, $P < 0.05$, Mann-Whitney U-test). The most likely explanation therefore is a cost of early reproduction to be paid as reduced reproductive performance later in life. This explanation is strongly supported by the brood size manipulation where females with enlarged brood size laid smaller clutches later in life than those with a reduced or unmanipulated brood (Table 2, Fig. 2b,c).

One basic premise in Williams's ideas on the evolution of senescence is that there should be selection for high early fertility. This was true for age of first breeding (see above). Furthermore there is also selection for a large clutch size among one-year-old females (regression; $LRS = 0.19 \times \text{clutch size} - 0.35$, $F = 7.47$, $n = 349$, $P < 0.01$).

Our results demonstrate that there is a permanent effect of early reproductive effort, and that an increase in the rate of senescence for fertility is therefore induced. The mechanism of the effect is at present not clear; it is not due to risky reproduction through ecological risks, a mechanism defined by Partridge², because that should affect only survival. It is possible to imagine a mechanism of physiological exhaustion lasting from year to year such as, for example, delayed moult, migration and start

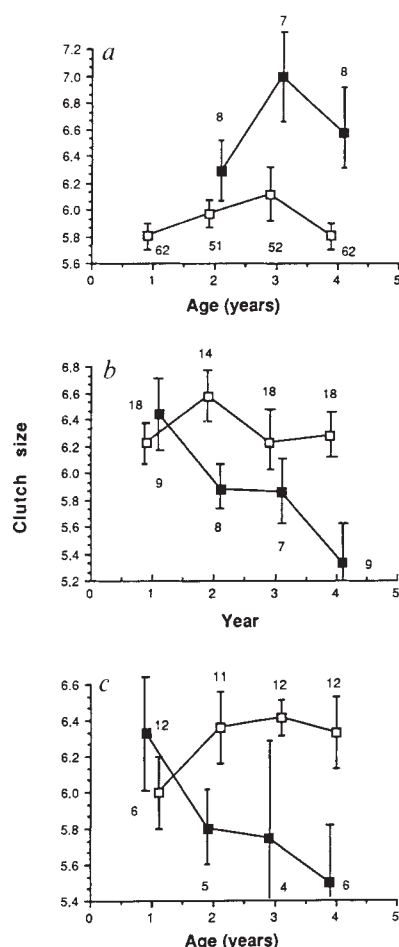


FIG. 2 Clutch size of collared flycatchers of different age *a*, using longitudinal data for females starting to breed as one (open) versus 2-year-olds (filled), *b*, females with enlarged (open) versus control broods (filled) irrespective of age at manipulation and *c*, females with enlarged (open) versus control broods (filled) manipulated as 1-year-olds. All data are ± 1 s.e.m. and numbers are sample sizes. Manipulations were done at year one in *b* and age 1 year in *c*. The earlier decline of clutch size for control individuals in *b* compared with *c* arises because many birds were here older than 1 year when manipulated. In fact age for this sample is ~ 4 years at three in *b*. This coincides with the start of senescence for fertility shown in Fig. 1 and in *a* and *c*. The displayed pattern cannot be due to between-year differences in clutch size as both groups were equally represented in all years. Also the changes in clutch size could not be explained by a change in date of egg laying because we could not find any differences in start of breeding later in life for our compared groups. The only significant differences were in the year following the manipulation, as birds from the enlarged group started, on average, to breed later than control birds (mean date in May 27.0 resp. 24.7 days, $t=2.21$, degrees of freedom=110, $P<0.05$). This difference, however, did not persist in the following years when clutch size differed even more between groups.

of breeding with lowered territory quality and breeding performance as a consequence. However, it is not obvious how that effect can linger on to later years.

A pleiotropic gene action, as proposed by Williams³, would involve an initial genetic effect, for example, on reproductive rate early in life, and a subsequent decline in reproductive probabilities achieved as lower fertility and/or survival. Our manipulation and the birds' decision about when to start breeding have mimicked the early genetic effect and the subsequent drop in clutch size that we observe would then presumably be caused by ordinary physiological effects of the early effort. Our results give support for Williams³ evolutionary explanations of senescence in that we demonstrate that when there is a strong

selection for an early high fertility in a natural population there is also a link between early effort and late performance. $\square$

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Clustering of L-type Ca^{2+} channels at the base of major dendrites in hippocampal pyramidal neurons

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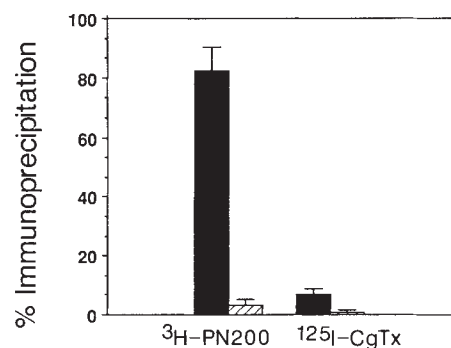
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INTEGRATION and processing of electrical signals in individual neurons depend critically on the spatial distribution of ion channels on the cell surface. In hippocampal pyramidal neurons, voltage-sensitive calcium channels have important roles in the control of Ca^{2+} -dependent cellular processes such as action potential generation^{1,2}, neurotransmitter release³, and epileptogenesis^{4,5}. Long-term potentiation of synaptic transmission in the hippocampal pyramidal cell, a form of neuronal plasticity that is thought to represent a cellular correlate of learning and memory^{6,7}, is dependent on Ca^{2+} entry mediated by synaptic activation of glutamate receptors that have a high affinity for NMDA (*N*-methyl-D-aspartate) and are located in distal dendrites^{8,9}. Stimuli causing long-term potentiation at these distal synapses also cause a large local increase in cytosolic Ca^{2+} in the proximal regions of dendrites¹⁰. This increase has been proposed to result from activation of voltage-gated Ca^{2+} channels¹⁰. At least four types of voltage-gated Ca^{2+} channels, designated N, L, T and P (refs 11, 12), may be involved in these processes. Here we show that L-type Ca^{2+} channels, visualized using a monoclonal antibody, are located in the cell bodies and proximal dendrites of hippocampal pyramidal cells and are clustered in high density at the base of major dendrites. We suggest that these high densities of L-type Ca^{2+} channels may serve to mediate Ca^{2+} entry into the pyramidal cell body and proximal dendrites in response to summed excitatory inputs to the distal dendrites and to initiate intracellular regulatory events in the cell body in response to the same synaptic inputs that cause long-term potentiation at distal dendritic synapses.

The different classes of voltage-gated Ca^{2+} channels in neurons have distinct physiological and pharmacological properties¹³. N-type Ca^{2+} channels are activated at relatively

FIG. 1 Immunoprecipitation of L- and N-type voltage-dependent Ca^{2+} channels by MANC-1 (■) and nonimmune IgG (▨). Specific binding sites for [^3H]PN200-110 (a dihydropyridine) and [^{125}I]-labelled CgTx, both prepared from rabbit hippocampal synaptic plasma membranes, immunoprecipitated to define L- and N-type channels, respectively.

METHODS. Hippocampal synaptic plasma membranes and L-type Ca^{2+} channels were prepared as previously described²⁶. For L-type channels, membranes were prelabelled with 2 nM [^3H]PN200-110 in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabelled PN200-110. The membranes were solubilized (2 mg ml^{-1} in 1% digitonin) and the extracts subjected to wheat-germ agglutinin-Sepharose chromatography. Peak fractions, containing the total and corresponding nonspecific binding, were saved and used for immunoprecipitation experiments. For N-type channels, hippocampal membranes were solubilized as above, diluted to a protein concentration of 0.2 mg ml^{-1} in 50 mM HEPES-Tris buffer, pH 7.4 (ref. 27) and divided into two equal samples. Vehicle or unlabelled CgTx (1 μM final) were added to define total and nonspecific binding, respectively, and allowed to incubate for 10 min at 22 °C. [^{125}I]-labelled CgTx (prepared as in ref. 28) was then added to both samples and allowed to incubate for 10 min at 22 °C. Aliquots from these postlabelled samples were then used in immunoprecipitation experiments. Immunoprecipitation of either [^3H]PN200-110 or [^{125}I]-labelled CgTx binding sites was performed as previously described²⁶. Each reaction contained 1 μM MANC-1 (purified from ascites using Protein-A Sepharose) or non-immune mouse IgG, 0.5–2.5 fmol [^3H]PN200-110 binding sites or 3,500 to 14,000 c.p.m. (1.0–4.3 fmol) of specifically bound [^{125}I]-labelled CgTx, 0.1% digitonin, 75 mM NaCl, 25 mM HEPES-Tris buffer, pH 7.4, 50 mM NaH_2PO_4 , 20 mM NaF, 2.5 mM NaEDTA, and protease inhibitors (aprotinin, 2 $\mu\text{g ml}^{-1}$; O-phenanthroline, 1 mg ml^{-1} ; pepstatin A 1 $\mu\text{g ml}^{-1}$;



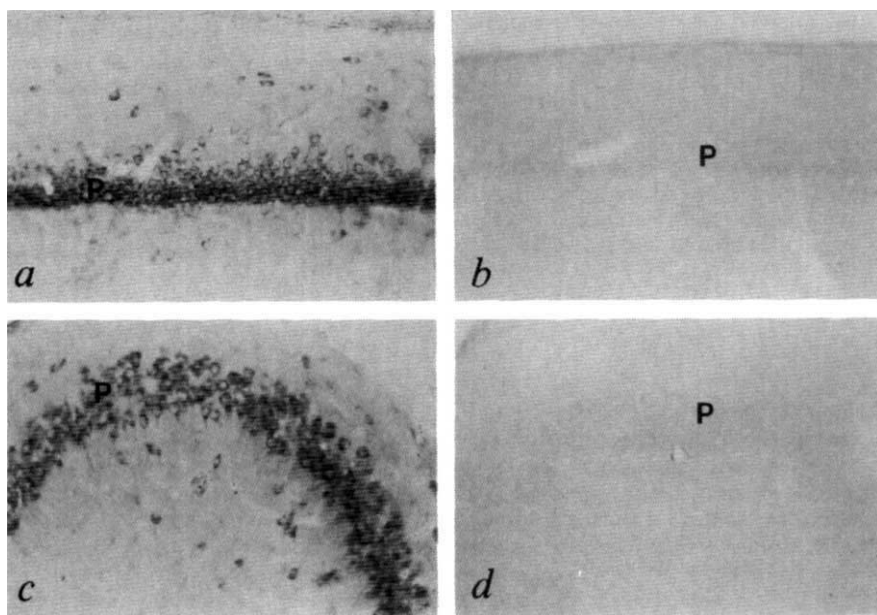
benzamide, 1 mg ml^{-1} and leupeptin, 1 $\mu\text{g ml}^{-1}$). This mixture was incubated at 4 °C for 4 h and 100 μl of 10% Protein-A Sepharose—which had been pre-equilibrated with rabbit versus mouse IgG and BSA, and washed with the above buffer—were added for 1–2 h at 4 °C. The mixture was centrifuged and the supernatant collected. The pellet was washed twice with the above buffer and these supernatants were also collected and pooled. [^{125}I]-labelled CgTx or [^3H]PN200-110 binding sites remaining in the supernatant after immunoprecipitation were precipitated by polyethylene glycol²⁹. The total amount of labelled Ca^{2+} channel in assays was defined as the sum of the specifically bound c.p.m. immunoprecipitated plus the remaining specifically bound c.p.m. precipitated by polyethylene-glycol from the supernatant.

positive voltages, can mediate both transient and sustained Ca^{2+} currents, and are inhibited potently by ω -conotoxin¹⁴. L-type Ca^{2+} channels are also activated at relatively positive voltages, mediate long-lasting Ca^{2+} currents, and are inhibited potently by dihydropyridines and only weakly by ω -conotoxin^{15,16}. The monoclonal antibody MANC-1 recognizes the α_2 subunit of skeletal muscle dihydropyridine-sensitive L-type Ca^{2+} channels and immunoprecipitates 95.3 ± 1.3% of dihydropyridine-sensitive Ca^{2+} channels from rabbit skeletal muscle that have been prelabelled with [^3H]PN200-110. It also immunoprecipitates 65% of the L-type Ca^{2+} channels labelled with [^3H]PN200-110 from rabbit brain, and analysis of the immunoprecipitates reveals α_1 , α_2 , and β -like subunits of brain L-type Ca^{2+} chan-

nels¹⁷. To determine whether MANC-1 specifically recognizes L- or N-type Ca^{2+} channels in the rabbit hippocampus, Ca^{2+} channels in synaptic plasma membranes from this region were prelabelled with [^3H]PN200-110 or [^{125}I] ω -conotoxin (CgTx), solubilized with digitonin, and immunoprecipitated with the antibody. MANC-1 immunoprecipitated 82.5 ± 7.8% ($n = 6$) of the dihydropyridine-sensitive, L-type Ca^{2+} channels from synaptic plasma membrane preparations prelabelled with [^3H]PN200-110; preimmune mouse IgG immunoprecipitated 3.4 ± 1.7% (Fig. 1). In contrast, only 6.9 ± 2.0% ($n = 4$) of the specifically bound [^{125}I]-labelled CgTx was immunoprecipitated by MANC-1 (IgG, 0.9 ± 0.6%). Most of this small amount of specifically bound [^{125}I]-labelled CgTx that is immunoprecipitated probably

FIG. 2 Localization of L-type voltage-sensitive Ca^{2+} channels in hippocampal pyramidal neurons and interneurons. *a*, Sagittal section of CA1 region stained with MANC-1 (magnification ×102). *b*, Control section of CA1 region illustrating lack of staining, especially the pyramidal layer (P), when MANC-1 was preincubated with purified Ca^{2+} channels before tissue staining (magnification ×102). Sagittal section of CA2–CA3 region demonstrating neurons stained with MANC-1 in strata oriens, pyramidal (P) and radiatum (magnification ×90). *d*, Control section of CA1 region (magnification ×102).

METHODS. Adult Sprague-Dawley rats ($n = 11$) were anaesthetized with sodium pentobarbitol and intracardially perfused with solution of 4% paraformaldehyde³⁰. The brains were immediately removed from the cranium, postfixed overnight, successively sunk in 10% (8 h), 20% (12 h) and 30% (48 h) solutions (w/v) of sucrose in phosphate buffer³⁰ at 4 °C and then sagittal or coronal (35 μm) sections were cut on a sliding microtome. Using the indirect peroxidase-antiperoxidase method, free floating sections were processed for immunocytochemistry as previously reported³⁰. All solutions were the same except 0.1% Triton X-100 was replaced by 0.1% digitonin. In brief, sections were incubated in the following antisera: 1, MANC-1 IgG purified by chromatography on Protein-A Sepharose and diluted 1:20, for 36 h at 4 °C; 2, goat anti-mouse IgG diluted 1:30 for 1 h at 37 °C; and 3, mouse PAP diluted 1:100 for 1 h at 37 °C. Sections were then reacted with 0.4% 3,3'-diaminobenzidine tetrahydrochloride and 0.003% H_2O_2 and then moun-



ted, dehydrated, covered with a coverslip and viewed. Control sections included replacing primary antisera with either normal mouse serum or buffer, and preincubating the MANC-1 antibody with purified skeletal muscle Ca^{2+} channel.

represents low affinity binding to L-type Ca^{2+} channels¹⁴. Thus, these results indicate that MANC-1 specifically recognizes dihydropyridine-sensitive L-type Ca^{2+} channels in the hippocampus.

Immunocytochemical studies using MANC-1 reveal that L-type Ca^{2+} channels are present in neurons located throughout the entire hippocampal region. Figure 2*a* and *c* illustrates cell bodies of pyramidal neurons and interneurons stained with MANC-1 in the strata oriens, pyramidale, and radiatum of the CA1 and the CA2-CA3 regions. Regions of the hippocampus containing axonal tracts are unstained. As a control, MANC-1 was preabsorbed with purified skeletal muscle Ca^{2+} channel before incubation with the tissue sections; MANC-1 staining was completely absent (Fig. 2*b*, *d*). Similar results are obtained in other control sections where MANC-1 was replaced by normal mouse serum or buffer.

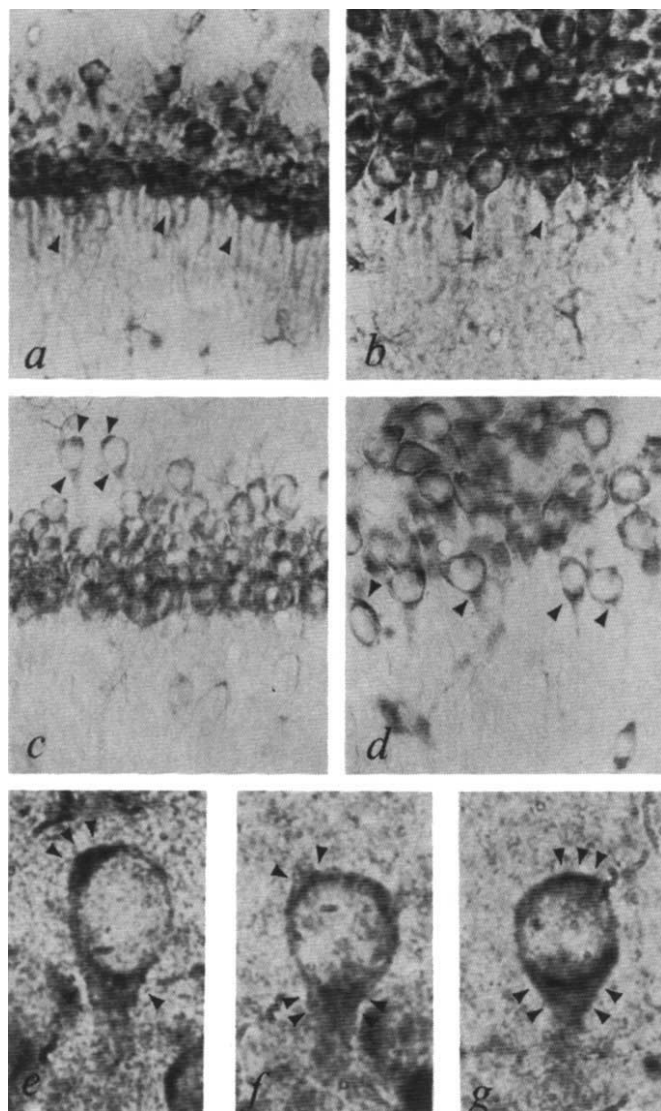


FIG. 3 Cellular distribution of L-type Ca^{2+} channels on hippocampal pyramidal neurons. *a*, *b*, Transverse sections of CA1 and CA2-CA3, respectively, stained using MANC-1. These panels illustrate L-type Ca^{2+} channels in the cell bodies and proximal portions of apical dendrites (arrowheads) of hippocampal pyramidal cells (*a*, $\times 448$; *b*, $\times 384$). *c*, *d*, Sagittal sections of CA1 and CA2-CA3, respectively. Dense MANC-1 staining is observed at the base of apical and basal dendrites (arrowheads) and in the proximal portion of apical dendrites (*c*, $\times 246$; *d*, $\times 266$). *e*-*g*, Higher magnifications of individual neurons illustrating dense immunoreactivity at base of dendrites (arrowheads) and around the periphery of the soma (*e*-*g*, $\times 1,100$).

Dendrites immunoreactive for L-type Ca^{2+} channels are observed associated with both pyramidal neurons and interneurons (Fig. 3). The apical dendrites of pyramidal neurons located in the CA1 and in the CA2-CA3 regions that stain with MANC-1 are most distinct when viewed in coronal sections (Fig. 3*a*, *b*, arrowheads), but are also observed in sagittal sections (Fig. 3*c*, *d*). When viewed in either coronal or sagittal sections, MANC-1 staining only extends along the proximal portion of these dendrites. Measurements made from the centre of the cell body indicate that MANC-1 immunoreactivity extends 17-50 μm (mean = 35 μm , $n = 30$) along the apical dendrites of CA1 and CA2-CA3 cells. These results suggest a discrete localization of these L-type Ca^{2+} channels in the cell bodies and proximal dendrites of hippocampal pyramidal cells.

Close examination of the pattern of immunostaining of the cell bodies of solitary pyramidal cells reveals that the L-type Ca^{2+} channels recognized by MANC-1 are clustered at the base of the apical and basal dendrites of the pyramidal cells (Fig. 3*c*, *d*, arrowheads). At higher magnifications (Fig. 3*e*-*g*), L-type Ca^{2+} channels seem to be located in a thin band around the periphery of the cell soma with relatively denser staining at sites where basal and apical dendrites originate from the cell body (arrowheads). These regions of clustering of L-type Ca^{2+} channels are generally located 5-10 μm ($n = 30$) from the centre of the cell body. Immunoreactivity for L-type Ca^{2+} channels is always observed along the proximal region of the apical dendrites, but does not usually extend for a measurable distance along the basal dendrites (Fig. 3*a*-*g*).

Previous physiological studies have shown that Ca^{2+} channels that mediate long-lasting, high threshold Ca^{2+} currents are present in the dendrites^{1,18} and in the somata¹⁹ of hippocampal neurons. Our results with an antibody that recognizes 82.5% of L-type Ca^{2+} channels in the hippocampus define the distribution of these L-type channels on the surface of both the cell body and the proximal dendrites of CA1-3 pyramidal neurons. These channels are expected to contribute to the high threshold Ca^{2+} currents measured in these regions. The high density of L-type Ca^{2+} channels at the base of dendrites suggests that they may mediate large local Ca^{2+} currents at that site. L-type Ca^{2+} channels have also been detected in the dendritic fields of the hippocampal pyramidal cells by receptor autoradiography with radiolabelled dihydropyridines^{20,21}, and N-type Ca^{2+} channels have been observed on the cell bodies of individual hippocampal neurons in cell culture with fluorescent derivatives of ω -CgTx (ref. 22). N-type Ca^{2+} channels and L-type Ca^{2+} channels that are not recognized by our antibody may be localized on distal dendrites and may contribute to the Ca^{2+} -dependent action potentials and intracellular Ca^{2+} accumulation observed there.

The localization of L-type Ca^{2+} channels in proximal dendrites of the pyramidal cells and the clustering of L-type Ca^{2+} channels at the base of their major dendrites are provocative findings in light of recent observations on the role of voltage-gated Ca^{2+} channels in intracellular calcium transients. The spatial distribution of intracellular Ca^{2+} accumulation in CA1 pyramidal cells induced by stimulation of the distal dendrites has a marked peak in the proximal dendrites¹⁰. These results led to the suggestion that voltage-gated Ca^{2+} channels may be unevenly distributed along dendrites, with a higher density of Ca^{2+} channels in proximal regions¹⁰. Our results provide direct evidence for such an uneven distribution of L-type Ca^{2+} channels and, in addition, show that these channels are clustered at the base of the dendrites of the pyramidal cells. We conclude that L-type Ca^{2+} channels that we have observed may be responsible for the sharp peak in Ca^{2+} accumulation in the proximal dendrites¹⁰. Other types of voltage-gated Ca^{2+} channels that are not recognized by our antibodies may also contribute to the sharp peak of Ca^{2+} accumulation in the proximal dendrites because the distribution of L-type Ca^{2+} channels described here does not precisely overlap the previously recorded intracellular Ca^{2+} transients¹⁰. However, when comparing our findings with

those from Ca^{2+} imaging¹⁰ surface-to-volume ratios must also be taken into consideration. In the cell body, entering Ca^{2+} is diluted into a large internal volume relative to surface area. In contrast, proximal dendrites have a much smaller internal volume relative to their surface area. For an equal rate of Ca^{2+} entry, the increase in cytosolic Ca^{2+} will be much larger in the proximal dendrites than in the soma. Similarly, because the diameter of the dendrites is smaller at more distal sites, the peak of cytosolic Ca^{2+} should be displaced to a more distal position than the peak of Ca^{2+} channel density, as we have observed. Thus, the distribution of L-type Ca^{2+} channels in the proximal dendrites and soma is consistent with a causal role in mediating cytosolic Ca^{2+} transients in response to stimulation of the distal dendritic tree.

Calcium entry through the NMDA-specific class of excitatory amino-acid receptors in synapses on distal dendrites induces long-term potentiation in CA1 pyramidal cells^{8,23-25}. We suggest that Ca^{2+} entry through L-type Ca^{2+} channels clustered in proximal dendrites and soma is activated by these same synaptic inputs and serves to initiate other intracellular regulatory events. These events may include Ca^{2+} -dependent protein phosphorylation and activation of gene expression. Thus, these strikingly localized L-type voltage-gated Ca^{2+} channels may serve as critical elements in neural integration and signal processing at the single cell level. □

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Anion channels activated by adrenaline in cardiac myocytes

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In heart cells, the catecholamine-activated cyclic AMP system regulates calcium¹ and potassium²⁻⁴ channels. We report here a novel class of chloride channels that can be activated by adrenaline in mammalian ventricular cells. Like the agonist-activated Cl^- channel currents of airway⁵⁻⁹ and colonic^{10,11} epithelial cells, the cardiac Cl^- -channel current shows outward rectification. But the unit conductance of cardiac Cl^- channels is smaller than that of epithelial Cl^- channels. The cardiac Cl^- channel is functionally voltage-independent, in contrast to the Cl^- channel in colonic epithelial cells^{10,11}. This channel could be responsible for the β -catecholamine-induced increase in cardiac membrane conductance¹² that has been attributed to activation of a Cl^- current¹³⁻¹⁵. Thus, sympathetic control of cardiac electrical activity involves not only the voltage-dependent, excitation-related cation channels, but also anion channels that generate a steady current.

Activation of channel currents in a cell-attached patch was observed when adrenaline was added to the bath (Fig. 1a). The patch membrane potential was clamped at a voltage 40 mV more negative than the resting potential (RP -40 mV). Compared to the usual delay of about 15 s for activation, the channel currents were deactivated more slowly by removing adrenaline, which seems consistent with involvement of intracellular metabolic pathways. We observed such adrenaline-activated channel currents in less than 5% of cell-attached patch recordings. The low success rate, which limited the number of cases analysed, is partly due to low channel density (see below).

Like other catecholamine-dependent ion channels in heart cells¹⁻⁴, the channels seemed to be regulated by cyclic AMP. Thus, channel currents with conductance and kinetics similar to those activated by adrenaline can be induced by exposing the cell to a high concentration (0.5 mM) of dibutyl cAMP (db-cAMP). Figure 1b shows an example of such currents.

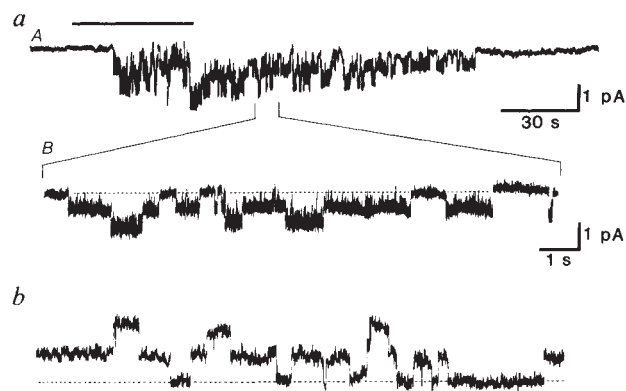
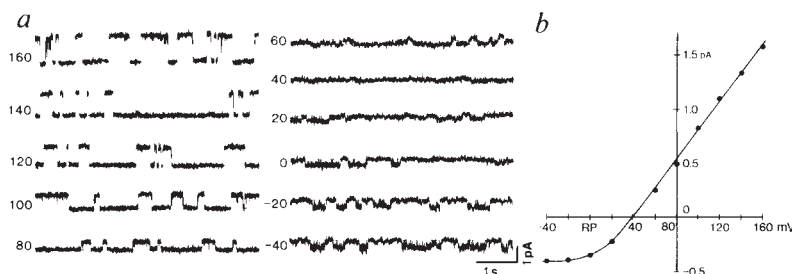


FIG. 1 Cell-attached patch recordings of channel activity induced by adrenaline or dibutyl cyclic AMP (db-cAMP). The pipette contained NaCl saline. *a*, Induction of channel activity by adrenaline. The patch membrane was clamped at a voltage 40 mV more negative than the resting potential (RP -40 mV), the latter being estimated to be -70 to -80 mV. Adrenaline (5 μM) was added to the bath (bar). *A*, Chart record of channel currents with low-pass filtration at 0.25 kHz; *B*, Expanded record of a part of *A*, low-pass filtered at 1 kHz. In this patch three channels seemed to be activated by adrenaline. Dotted line indicates closed level. In this experiment, the cell was briefly exposed to adrenaline three times, and channel currents of the type shown were repeatedly observed. *b*, Channel activity in a cell treated with db-cAMP. Record of outward channel currents at RP +120 mV, low-pass filtered at 0.5 kHz. Two channels were active in this patch. Calibration in *aB* also applies to *b*. In this type of experiment, cells were exposed to 0.5 mM db-cAMP for at least 10 min before formation of the patch. In three out of 71 such patches, channel activity was detected.

METHODS. Single cells were isolated from guinea-pig heart ventricles using collagenase^{28,29}. Single channel currents were recorded from cell-attached membrane patches³⁰. The patch electrode had a resistance of 3-5 M Ω when filled with pipette solution. The bath solution was Tyrode solution containing NaCl 140 mM, KCl 5.4 mM, CaCl_2 1.8 mM, MgCl_2 1 mM, glucose 5.5 mM and HEPES-NaOH 5 mM (pH 7.4). Adrenaline (1-5 μM) and db-cAMP (0.5 mM) were applied to the cells by adding these agents to the bath solution. Three types of pipette solution were used. The NaCl saline contained NaCl 140 mM, CaCl_2 1.8 mM, MgCl_2 1 mM and HEPES-NaOH 5 mM (pH 7.4). This solution also contained 2 mM BaCl_2 to block K^+ channels. The high-NaCl saline was prepared by adding 150 mM extra NaCl to the NaCl saline. The low-Cl NaNO_3 saline was made by replacing 130 mM NaCl in the NaCl saline with 130 mM NaNO_3 . Experiments were performed at 34-36 $^{\circ}\text{C}$.

FIG. 2 Adrenaline-activated Cl^- channels at various membrane potentials in a cell-attached patch. The patch membrane potential was varied while adrenaline-induced channel activity was seen. The pipette contained NaCl saline (149.6 mM Cl^-). *a*, original records of single channel currents, filtered at 0.5 kHz. Adrenaline concentration, 5 μM . Numbers at the left of each trace indicate deviation (in mV) from the resting potential. *b*, relationship between amplitude of single channel current (ordinate) and membrane potential (abscissa), obtained from the data shown in *a*. Membrane potentials are expressed as in *a*. The single channel conductance for the outward currents was 13 pS, and the reversal potential was $\text{RP} + 40$ mV.



Unlike the inward currents, outward currents through these channels showed no bursting activity.

Figure 2 shows original recordings of the adrenaline-activated channel currents and their I - V relation. The I - V relation of the outward currents seemed to be linear, but the inward currents showed a trend of saturation with hyperpolarizations (outward rectification). The single-channel conductance for outward currents was 13.1 ± 1.2 pS (mean $\pm$ s.d., $n = 4$), and the reversal potential, estimated from I - V relation, was $\text{RP} + 37 \pm 4$ mV. The outward rectification was generally present, but its degree was variable (see also Fig. 3c). If the cell membrane potential during adrenaline application is -80 to -70 mV¹², the absolute reversal potential of channel currents may be -43 to -33 mV. These values do not match the equilibrium potential of any principal cations present inside and outside the cells, but appear to be close to the Cl^- equilibrium potential (-60 to -40 mV) calculated using the reported cardiac intracellular Cl^- activity¹⁶⁻²⁰.

Cl^- selectivity of the channel was further tested by nearly doubling the NaCl concentration in the pipette. If the channel is Cl^- selective, a more negative reversal potential and an

increased outward channel conductance should be recorded, whereas a positive shift of the reversal potential should occur for Na- or cation-selectivity. The reversal potential obtained with the high-NaCl saline (estimated to be $\text{RP} + 4$ mV) was more negative than that obtained before, and the conductance for outward currents was increased to 20 pS (Fig. 3a, c). In two similar experiments, the reversal potential was $\text{RP} + 25$ and $\text{RP} + 21$ mV, and the conductance was 16 and 17 pS. We conclude that the adrenaline-activated channel is Cl^- selective. The variation in the reversal potential may be attributed to variations in the resting potential and/or variations of intracellular Cl^- concentration in individual cells. When the pipette contained low- Cl^- NaNO_3 saline (Fig. 3b, c), channel activities similar to those obtained with NaCl saline were observed. NO_3^- ions have been suggested to support anion currents in cardiac tissue^{21,22} as well as in other excitable tissues²³.

The channel showed characteristic kinetics. The open-time histogram of outward currents was fitted to a single exponential function with a long mean open-time of 200–700 ms ($n = 4$, Fig. 4a). The inward currents showed high-frequency closings during the burst (Figs 1a, 2a and 3a, b), indicating presence of an additional fast-gating process or some blocking phenomena²³. The mean burst-time, obtained for inward currents by neglecting the short (< 2 ms) closings, was in the same order as the mean open-time for outward currents. Although different in different patches, the mean open-time and burst-time, and open probability (P_o) (Fig. 4b), showed no systematic variation with voltage.

Our results provide direct evidence, for intact cardiac membrane, that β -adrenoceptor-activated Cl^- current recorded under whole-cell conditions¹³⁻¹⁵ flows through ion channels

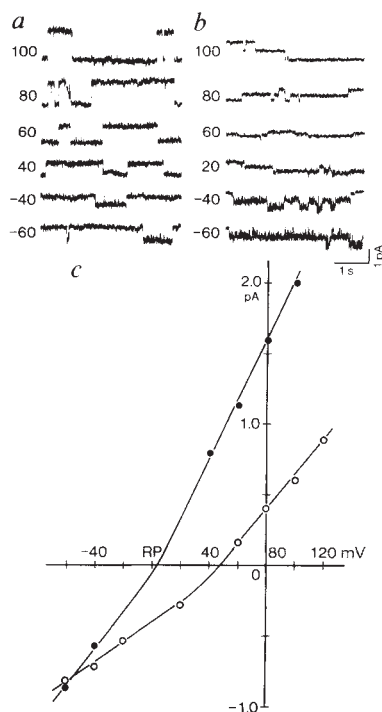


FIG. 3 Adrenaline-activated Cl^- channels with different pipette solutions. The pipette contained either high-NaCl saline (299.6 mM Cl^- , *a*) or low- Cl^- NaNO_3 saline (130 mM NO_3^- + 19.6 mM Cl^- , *b*). *a* and *b*, Original records of single channel currents at various membrane potentials, filtered at 1 kHz. Adrenaline concentration, 5 μM . Two channels were active in *b*. *c*, I - V relation of the channel current obtained with high-NaCl saline ($\bullet$) and low- Cl^- NaNO_3 saline ($\circ$). Details are as in Fig. 2.

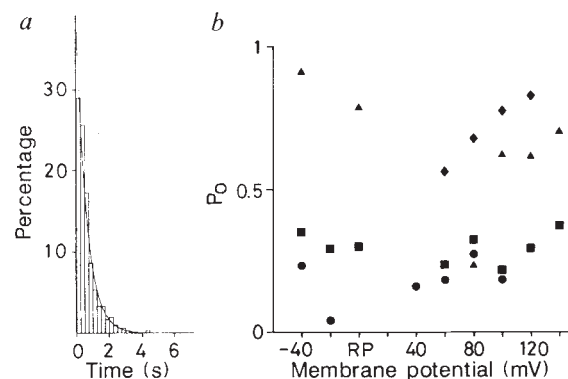


FIG. 4 *a*, Open-time histogram obtained at $\text{RP} + 120$ mV. Number of events sampled was 298. Time bins are 250 ms. The histogram was fitted to a single exponential with a time constant of 637 ms. The channel was activated by 5 μM adrenaline. *b*, Open probability of the channel (P_o , calculated as a fraction of time for which the channel stays open), as a function of membrane potential (abscissa). Membrane potentials are expressed as amounts of deviation from the resting potential (RP). The channels were all activated by 5 μM adrenaline. P_o was calculated from the data filtered at 1 kHz. For -40 to 0 mV, at which potentials the currents were inward and showed high-frequency closings during the burst, closing gaps shorter than 2 ms were neglected for calculation. Data from four patches (different symbols).

rather than an electrogenic cotransporter²⁴. Unitary Cl^- currents have been recorded in cardiac membrane vesicles incorporated into lipid bilayers²⁵. But the vesicular Cl^- channels are active without catecholamine and their conductance is much larger (55 pS), suggesting a different kind of Cl^- channel. The cardiac Cl^- channels reported here have properties different from the agonist-activated Cl^- channels in epithelial cells. The unit conductance of cardiac Cl^- channels is smaller than that of epithelial Cl^- channels (25 to 50 pS)⁵⁻¹¹. The cardiac Cl^- channel is practically voltage-independent, in contrast to the Cl^- channel in colonic epithelial cells^{10,11}. Detailed voltage-dependence is not known in the Cl^- channels of other types of epithelial cells.

In our previous study¹⁵, adrenaline increased membrane conductance by 10 nS in a whole cell. If the open probability of the channel is assumed to be 0.5, the 10 nS increase in conductance is produced by about 1,600 channels. This leads to estimation of a low channel density (about $0.1 \mu\text{m}^{-2}$) using average cell surface area of $14,000 \mu\text{m}^2$, the latter value being obtained from capacitance measurements of ventricular cells^{15,26} with an assumption of specific membrane capacitance of $1 \mu\text{F cm}^{-2}$.

Activation of the anion channel by β -adrenergic stimulation should accelerate action potential repolarization in cardiac muscle, associated with increased heart rate. The Cl^- channel may also be involved in the natural pacemaker mechanism. The channel activated by catecholamine should increase background inward currents at the diastolic potential level, which would produce an accelerated pacemaker rhythm.

As whole-cell studies demonstrate that activation of cardiac Cl^- current by β -adrenoceptor stimulation is mediated by cAMP-dependent protein kinase^{13,14,27}, it is likely that the same mechanism is involved in the activation of the anion-channel currents described here. Induction of similar channel currents by db-cAMP supports this view. If so, cAMP-dependent protein kinase regulates, in heart muscle membrane, not only voltage-dependent, excitation-linked cation channels such as the Ca^{2+} channel¹ and the delayed rectifier K^+ channel²⁻⁴, but also anion channels that generate a steady current. Studies on excised patches should allow detailed investigation of intracellular regulatory mechanisms of these channels, with improved experimental control. □

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Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen

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EFFECTOR T cells are restricted to recognizing antigens associated with major histocompatibility complex (MHC) molecules¹. Specific recognition is mediated by the $\alpha\beta$ heterodimer of the T-cell receptor (TCR)/CD3 complex^{2,3}, although other membrane components are involved in T-cell antigen recognition and functions. There has been much controversy in this regard over the part played by the CD4 glycoprotein. It is known that expression of CD4 correlates closely with the cell's ability to recognize antigens bound to class II MHC molecules^{4,5} and that CD4 can bind to class II molecules⁶. Also monoclonal antibodies to CD4 can modify signals generated through the TCR/CD3 complex⁷⁻¹⁰. It has therefore been proposed that CD4 binds to class II molecules, coaggregates with the TCR-CD3 complex and aids the activation of T cells. But given that TCR can itself impart restriction on the cell, it remains unclear whether the contribution of CD4-derived signals to those generated through the TCR $\alpha\beta$ -CD3 complex is central to this activation. Here we report that when preceded by ligation of CD4, signalling through TCR $\alpha\beta$ results in T cell unresponsiveness due to the induction of activation dependent cell death by apoptosis. These results imply that CD4 is critically involved in determining the outcome of signals generated through TCR, and could explain why the induction of effector T cells needs to be MHC-restricted.

Monoclonal antibodies to TCR $\alpha\beta$ (H57.597, ref. 11) stimulated DNA synthesis by splenic T cells bearing the CD4 antigen in the presence of T cell-depleted irradiated splenocytes. The level of thymidine incorporation induced by the antibodies was maximal over a broad concentration range (Fig. 1), titrating to background between 1-10 ng ml⁻¹. Pretreatment with antibodies to CD4 inhibited the ability of the anti-TCR $\alpha\beta$ antibodies to induce DNA synthesis (Fig. 1). Two monoclonal antibodies to CD4 (H129, ref. 12; GK 1.5, ref. 13) had the same effect and both required crosslinking with polyclonal antiserum (data not shown).

By contrast, the response to antibodies to CD3 ϵ (145.2C11, ref. 14) was virtually unaffected by pretreatment with anti-CD4 antibodies (Fig. 1). The maximum inhibition was 40%, occurring only at the lowest concentration of the stimulating antibody (Fig. 1), and the level of thymidine incorporated was consistently higher than that induced by antibodies to TCR $\alpha\beta$. This differential capacity to signal through TCR $\alpha\beta$ and CD3 ϵ has been reported previously in other systems¹⁵ and may reflect either differences in antibody affinities or a functional uncoupling of TCR $\alpha\beta$ from CD3 components^{15,16}. The differential effects of anti-CD4 antibodies in perturbing signals generated through TCR $\alpha\beta$ and CD3 ϵ supports the latter hypothesis (see below). To determine whether membrane-associated molecules other than CD4 could alter signals generated through TCR $\alpha\beta$, we analysed the effects of crosslinking MHC class I, LFA-I and CD2 to TCR $\alpha\beta$ -CD3 by treatment with the appropriate antibodies. Cell surface expression of these molecules was easily detectable by flow cytometry (data not shown). Crosslinking did not affect levels of thymidine incorporation induced either by anti-TCR $\alpha\beta$ or anti-CD3 ϵ antibodies (Table 1).

The inability to induce DNA synthesis through TCR $\alpha\beta$ on cells pretreated with anti-CD4 antibodies was not due to

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TABLE 1 Specificity of CD4-mediated inhibition

Stimulus	CD4	% of control response after pretreatment with antibodies against:		
		MHC class I	LFA-1	CD2
Anti-TCR $\alpha\beta$	13	101	100	93
Anti-CD3 ϵ	109	148	112	113

T cells carrying the CD4 antigen were prepared as described in Fig. 1. Cells were pretreated at 10^7 ml^{-1} with either anti-CD4 (H129), anti-MHC class I (M142, ref. 32), anti-CD2 (12-15, ref. 33), or anti-LFA-1 (I21/7.7, ref. 34) each at $50 \mu\text{g ml}^{-1}$ for 45 min at room temperature, as described in Fig. 1. Treated cells were cultured with the indicated stimuli at 100 ng ml^{-1} and collected as described in Fig. 1.

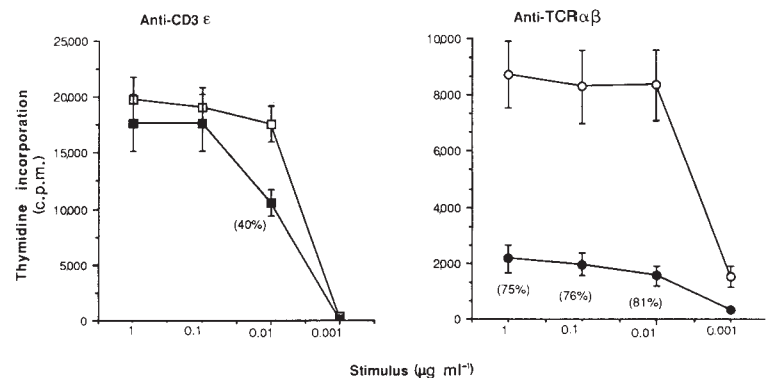
comodulation of TCR-CD3 with CD4^{17,18}. Levels of TCR $\alpha\beta$ expression, determined flow cytometrically, were unaffected by pretreatment with anti-CD4 antibodies (data not shown). The pretreatment did not alter the ability of anti-TCR $\alpha\beta$ antibodies to induce increases in intracellular free Ca^{2+} concentrations, and neither the frequency of responding cells nor the amplitude of the induced Ca^{2+} signal was affected (data not shown). The

FIG. 1 CD4-mediated inhibition of anti-TCR $\alpha\beta$ and anti-CD3 ϵ induced stimulation. The stimulation of CD4⁺ splenic T cells was quantitated by measuring antibody-mediated [³H]thymidine incorporation by T cells (3×10^4) in the presence of irradiated T cell-depleted spleen cells (5×10^5). Splenic CD4⁺ T cells were prepared from BALB/c mice (Charles River, Montreal, Quebec). B cells were depleted by passage of splenocytes over immunoglobulin-anti-immunoglobulin columns (Beckman) and the resulting population treated with anti-CD8 antibody (3.168, ref. 35) plus complement. The remaining viable cells were separated on discontinuous percoll gradients. Those cells binding at the $\rho = 1.109/1.079$ interface were used throughout. These cell preparations were consistently >95% positive for Thy-1.2, TCR-CD3 and CD4. They were <1% positive for mlg, class II and CD8. Filler cells were prepared by treating syngeneic splenocytes simultaneously with anti-Thy-1.2, anti-CD4 and anti-CD8 in the presence of complement. Viable cells were isolated on discontinuous percoll gradients ($\rho = 1.109/1.066$) before gamma irradiation (2,000 rad). T cells carrying the CD4 antigen were pretreated or not at 10^7 ml^{-1} with $50 \mu\text{g ml}^{-1}$ H129 or GK1.5 for 45 min at room temperature, washed, and bound antibodies crosslinked with rabbit anti-mouse immunoglobulin at $50 \mu\text{g ml}^{-1}$ for 45 min at 37 °C. Following this treatment the cells were washed and plated with fillers and the indicated concentrations of 145.2C11 and H57.597. After 36 h of culture in Iscove's modified Dulbecco's medium (IMDM) with no serum supplements, each culture was pulsed

inhibition of DNA synthesis and the lack of inhibition of an early signalling event suggests that pretreatment with antibodies to CD4 alters, rather than ablates, signalling through TCR $\alpha\beta$.

Apoptosis is a form of cell death characterized by the generation of nucleosome-sized fragments as a result of the activation of an endogenous endonuclease that requires Ca^{2+} (ref. 19). Apoptosis mediated by TCR-CD3 ϵ occurs in thymocytes²⁰⁻²², as well as in hybridomas derived from mature T cells²³. As pretreatment with anti-CD4 antibodies does not inhibit the ability of anti-TCR $\alpha\beta$ to induce mobilization of intracellular Ca^{2+} , we addressed the possibility that cells pretreated with anti-CD4 antibodies die when stimulated through TCR $\alpha\beta$.

The induction of apoptosis was demonstrated by visualization of nucleosome-sized DNA fragments¹⁹ and quantitated by assessing the proportion of fragmented DNA²⁴. Overnight culture of T cells bearing the CD4 antigen with anti-TCR $\alpha\beta$ antibodies under crosslinking conditions induced a <5% increase in the level of fragmented DNA over the background level observed in the absence of anti-TCR $\alpha\beta$ antibodies (Fig. 2, lanes 1 and 2; Fig. 3). As previously reported for immature thymocytes²¹, anti-CD4 crosslinking in the absence of subsequent stimulation did not give levels of DNA fragmentation ($9.4 \pm 2.8\%$) significantly different from those obtained on stimulation



with $1 \mu\text{Ci}$ of [³H]thymidine and collected 6 h later. Numbers represent the mean and one standard error from a pool of 8 individual experiments. Numbers in parenthesis, where indicated, represent per cent inhibition compared with non-pretreated controls. Left panel, Anti-CD3 ϵ (145.2C11) induced thymidine incorporation before (□) and after (■) anti-CD4, rabbit anti-mouse immunoglobulin pretreatment. Right panel, Anti-TCR $\alpha\beta$ (H57.597) induced thymidine incorporation before (○) and after (●) anti-CD4 rabbit anti-mouse immunoglobulin pretreatment.

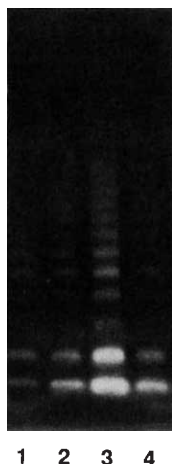


FIG. 2 Induction of apoptosis in peripheral CD4⁺ T cells. T cells carrying the CD4 antigen, pretreated or not as described in Fig. 1, were incubated at 10^7 ml^{-1} with anti-TCR $\alpha\beta$, anti-CD3 ϵ , and in some cases simultaneously with anti-CD4, each at $10 \mu\text{g ml}^{-1}$ for 45 min at room temperature. After washing, 1.3×10^6 cells were plated in 0.5 ml IMDM in 24-well Costar plates precoated with rabbit anti-mouse immunoglobulin at $50 \mu\text{g ml}^{-1}$. After overnight incubation, the cells were collected and the extent of DNA fragmentation determined by a modification of the method of Sellins and Cohen¹⁹. In brief, the cells were collected by centrifugation at 200g for 10 min. The pellet was lysed with 0.5 ml hypotonic lysing buffer, pH 7.5 (10 mM Tris buffer, 1 mM EDTA, 0.2% Triton X-100). The lysates were immediately centrifuged at 13,000g for 10 min and the supernatant, containing fragmented DNA, was collected. DNA from the 13,000g supernatants were precipitated overnight at -20°C in 50% isopropanol and 0.5 M NaCl. The precipitates were collected after centrifugation at 13,000g, air-dried, and resuspended in 10 mM Tris, 1 mM EDTA, pH 7.4. Loading buffer containing 15 mM EDTA, 2% SDS, 50% glycerol and 0.5% bromophenol blue was added to samples at 1:5 (v/v), and samples heated to 65°C for 10 min. Electrophoresis was in 0.75% agarose for 3 h at 90 V. DNA was visualized by staining with ethidium bromide, and photographed. Lane 1, untreated cells; lane 2, anti-TCR $\alpha\beta$; lane 3, pretreatment with anti-CD4 followed by anti-TCR $\alpha\beta$; lane 4, simultaneous addition of anti-CD4 and anti-TCR $\alpha\beta$.

with antibodies to TCR-CD3. Simultaneous exposure to anti-TCR $\alpha\beta$ and anti-CD4 antibodies under these conditions resulted in a slight decrease in the proportion of fragmented DNA (Fig. 2, lane 4; Fig. 3). By contrast, when cells were pretreated with anti-CD4 antibodies and then incubated overnight with anti-TCR $\alpha\beta$ antibodies, DNA fragmentation increased significantly, reaching levels up to 10 times those induced by simultaneous addition of anti-TCR $\alpha\beta$ and anti-CD4 antibodies (Fig. 2, lane 3; Fig. 3). We conclude that CD4 plays a critical part in determining the outcome of signals generated through TCR $\alpha\beta$.

In contrast to the results obtained with anti-TCR $\alpha\beta$, addition of anti-CD3 ϵ to cells pretreated with anti-CD4 did not increase the level of DNA fragmentation above that induced in the absence of anti-CD4 pretreatment (Fig. 3). These results suggest that TCR $\alpha\beta$ can be functionally uncoupled from some CD3 components. This could be explained if anti-TCR $\alpha\beta$ were to induce a series of progressive activation signals. The involvement of CD3 components in this progression could be preceded by several events which could be susceptible to CD4-mediated regulation. Those signals generated directly through some CD3 components, on the other hand, may not be. Hence, direct ligation of CD3 ϵ with antibody could functionally obviate upstream signals generated through TCR $\alpha\beta$. Alternatively, or in addition, CD3 could exist as functionally distinct signalling complexes, perhaps generated as a consequence of TCR-CD3 interaction with CD4. Signalling through these CD3 components would circumvent the requirements for TCR $\alpha\beta$ ligation. Our results predict that unless CD4 is incorporated in the TCR-CD3 complex, the generation of signals through TCR $\alpha\beta$ will result in cell death. Why then is there any T-cell activation on direct ligation of TCR $\alpha\beta$ *in vitro*? The requirement for oligomerization of TCR $\alpha\beta$ to induce activation signals^{15,16} could lead to the non-specific trapping of CD4 molecules. This inefficiency may account for the differential response to anti-TCR $\alpha\beta$ and anti-CD3 ϵ antibodies in the absence of anti-CD4 pretreatment (Fig. 1).

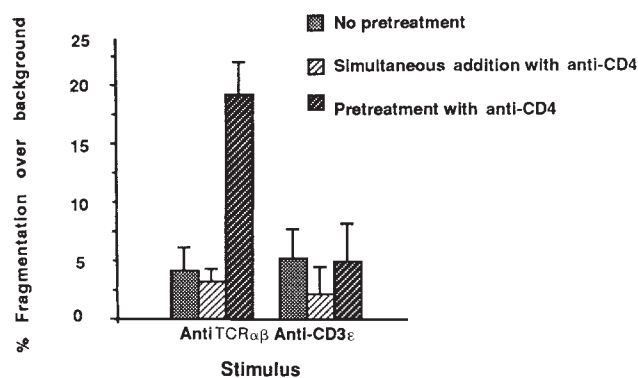


FIG. 3 DNA Quantitation. The DNA in the precipitates and supernatants generated from the 13,000g spin of lysed cells (Fig. 2, legend) was precipitated overnight at 4°C in 0.5 ml of lysis buffer and 0.125 ml of 50% trichloroacetic acid, and centrifuged at 13,000g for 4 min. The DNA was hydrolysed by heating to 90°C for 10 min in 240 μ l 5% trichloroacetic acid and quantitated by a modification of the diphenylamine method of Burton²⁴. In brief, 0.48 ml of DPA reagent [0.15 g DPA, 0.15 ml sulphuric acid, 0.05 ml acetaldehyde (16 mg ml⁻¹)/10 ml glacial acetic acid] was added to each tube. After a 48-h incubation at 4°C, absorbance at 570 nm was measured. Per cent fragmentation was calculated as the ratio of DNA in the 13,000g supernatants to the total DNA recovered in the supernatant and the pellet. Per cent fragmentation of immediately *ex vivo* CD4⁺T cells was 10.2%. Data is presented as % fragmentation over background which averaged to 25.92 $\pm$ 5.5 in eight experiments reported. Crosslinking of anti-CD4 in the absence of subsequent stimulation resulted in an average increase of 9.4 $\pm$ 2.8% fragmentation over background.

The mechanism through which CD4 mediates its effect is not clear. Whether the requirement for crosslinking reflects the need to decrease the proximity of CD4 and TCR-CD3, or to sterically hinder their interaction, remains unclear. In addition, given that crosslinking CD4 results in the activation of the tyrosine kinase p56^{lck} (ref. 25), inappropriately directed kinase activity could also be involved. A precedent for the effects of separate ligation of CD4 and TCR $\alpha\beta$ has been established *in vivo*. Pretreatment of adult animals with anti-CD4 antibodies abrogates rejection of primary allografts as well as the production of alloantibodies^{26,27}. Importantly, the results reported here are not easily reproducible using long-term propagated clones of CD4⁺, MHC class II-restricted T cells MHJ (unpublished observation). Differences between signalling mediated by CD4 and TCR-CD3 in immediately *ex vivo* T cells and clones are becoming evident. For example, crosslinking of CD4 with antibody (ref. 28, and MHJ unpublished observations) on cloned, but not *ex vivo* (MHJ unpublished observations) T cells, results in Ca²⁺ mobilization. It is possible that signal transduction machinery in cloned T cells is irreversibly locked into those pathways which, in fact, reflect our ability to propagate these cells indefinitely.

The demonstration that CD4 binds MHC class II molecules⁶, provides the mechanism through which it can interact with CD3 components associated with antigen/MHC class II-specific TCR molecules. Moreover, p56^{lck}, which physically associates with the cytoplasmic domain of CD4 provides a molecular basis through which the CD4 complex can modify CD3 components²⁹. The ζ chain is present in the CD3 complex either as a covalently associated homodimer or as a heterodimer with the η chain³⁰; experiments suggest that the consequences of signalling through each form is different³¹. Specifically, the ability to induce programmed cell death through TCR $\alpha\beta$ requires the presence of ζ - η heterodimers. On the basis of this and our results, one might speculate that association of CD4 with TCR-CD3 determines which CD3 components will function in TCR $\alpha\beta$ -mediated signalling. Therefore, the fate of a T cell would be determined by the involvement of CD4 in antigen recognition, which would, in turn, explain why T cell activation need be MHC restricted. □

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Different amino acids at position 57 of the HLA-DQ β chain associated with susceptibility and resistance to IgA deficiency

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THE human leukocyte antigens (HLA) are implicated in the genetic susceptibility to a large number of diseases. Some of the diseases associated with HLA class II are related to specific amino acids or epitopes of the domain of the HLA class II molecule that is distal to the membrane¹⁻³. In man, selective immunoglobulin A deficiency is the most common immunodeficiency, frequently resulting in recurrent sino-pulmonary infections and gastro-intestinal disorders. Associations have been described with HLA class I, and to a lesser extent with different class II alleles⁴⁻⁹, which might indicate that they share some common feature. Here we study 95 IgA-D patients and find positive associations with three DR-DQ haplotypes and a strong negative association with a fourth haplotype. Comparison of the sequences of the polymorphic amino-terminal domain of the DQ β chain showed that the three 'susceptibility' haplotypes all had a neutral alanine or valine at position 57. The 'protective' allele had the negatively charged aspartic acid at this position (Asp 57). Codon 57 of the HLA-DQ β chain has been implicated in the susceptibility to insulin-dependent diabetes mellitus^{1,2}. Our data suggest that the same amino acid position could possibly also influence susceptibility and resistance to selective immunoglobulin A deficiency.

First we confirmed the well-established DR3 association in selective immunoglobulin A deficiency (IgA-D). The DR3

specificity has recently been split and IgA-D was found to be associated with the DRw17 subtype. Positive associations with two additional DR-DQ haplotypes, DR7,DQw2 and DR1,DQw5 were observed, as well as a strong negative association with DRw15, DQw6 (Table 1). The increased relative risks (RR) conferred by the DRw17,DQw2 and DR7,DQw2 haplotypes were additive, whereas DR1,DQw5 acted synergistically with these two haplotypes: the RR of DRw17,DQw2/DR7,DQw2 heterozygous individuals being 8.9, whereas the RRs of DRw17, DQw2/DR1,DQw5 and DR7,DQw2/DR1,DQw5 heterozygotes were 37.3 and 17.2, respectively. This finding might indicate heterogeneity in the genetic susceptibility to IgA-D. Only 2 of 35 DR1,DQw5 positive IgA-D patients had anti-IgA antibodies (determined by haemagglutination) compared with 16 of 45 DRw17,DQw2 positive patients ($P < 0.005$), which supports this hypothesis.

Forty-seven IgA-D patients were HLA-DP typed by *Msp*I restriction fragment length polymorphism (RFLP) analysis¹⁰. No HLA-DP association was observed. The linkage disequilibrium between DRw17,DQw2 and DPw1 was not more pronounced in IgA-D than in controls. These findings suggest that the haplotypes associated with IgA-D are not extended centromerically throughout the entire HLA class II region. The associations with the HLA class I alleles B8 and B14 (refs 4-6) and with loci in the class III region¹¹ might indicate that the haplotypes associated with IgA-D are extended telomerically.

When looking for a shared feature between the observed DR-DQ associations, the published sequences of the polymorphic second exon of *DRB1*, *DQA1* and *DQB1* genes were compared. Analysis of *DRB1* and *DQA1* sequences were unrewarding. As can be seen in Table 2, comparison of the polymorphic residues of the HLA-DQ β chain in the IgA-D associated alleles were more suggestive. The three susceptibility alleles all had a neutral amino acid at position 57, alanine or valine, whereas the protective DRw15,DQw6 had the negatively charged aspartic acid. In addition, three *DQB1* alleles with neutral to negative associations with IgA-D were Asp 57 positive; *0301, *0402 and *0603. The importance of position 57 is further stressed by the observation that when individuals carrying the three DR-DQ haplotypes positively associated with IgA-D are removed from the patient group and the control group, 10 of the remaining 14 IgA-D patients are non-Asp 57 positive compared with 24 of the remaining 57 controls ($P < 0.05$). In the whole material, 62% of patients were HLA-DQ β non-Asp 57 homozygous compared with 22% of controls ($P < 10^{-7}$; RR 5.9). Only 4% of patients were Asp 57 homozygous compared with 34% of controls ($P < 10^{-5}$; RR 0.1) (Fig. 1). The

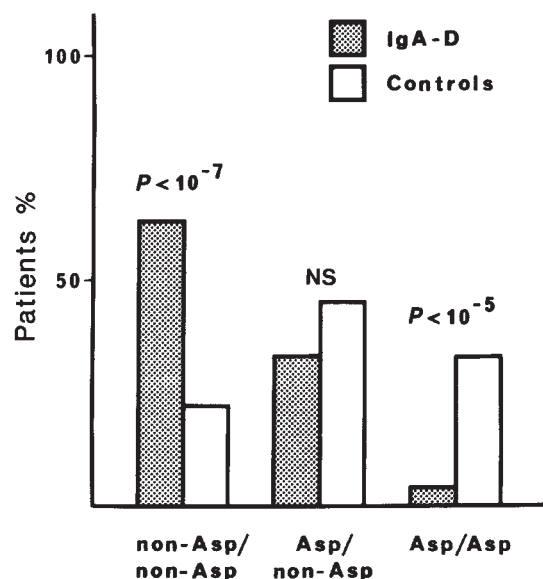


FIG. 1 The frequency of occurrence of Asp at position 57 of the HLA-DQ β chain in IgA-D patients compared with controls.

METHODS. All patients and controls were HLA-DR and -DQ typed by *Taq*I RFLP analysis²³⁻²⁵ and the amino acid at position 57 was inferred. In 72 of the 95 patients and 94 of the 100 controls Asp 57 and non-Asp 57 was confirmed by hybridizing the second exon of *DQB1* genes¹ (amplified by polymerase chain reaction) with 8 oligonucleotide probes centred around codon 57 (ref. 13). The two methods gave completely concordant results.

TABLE 1 Phenotype frequencies (%) of HLA-DR-DQ haplotypes in IgA-D patients* and healthy Swedish controls

Dw†	DR†	DQ†	Phenotype frequency IgA-D (n=95)	Controls (n=100)	P _c ‡	IgA-D association	Relative risk
1	1	w5	37	21	<0.005	positive	2.2
2	w15	w6	3	30	<0.001	negative	0.1
3	w17	w2	46	15	<0.0001	positive	4.9
17	7	w2	21	7	<0.02	positive	3.3

* IgA-D was defined as a serum level of IgA of less than 0.05 g l⁻¹. The IgA-D patients were unrelated and of Scandinavian extraction. One third of the patients had symptomatic IgA-D, in most cases manifested by recurrent sino-pulmonary infections.

† HLA-DR and -DQ typing was performed by *TaqI* RFLP analysis. For details of methodology and probes used see ref. 22. The serologically (DR and DQ) and cellularly (Dw) defined specificities associated with specific *TaqI* RFLP patterns²³⁻²⁵ are given in the text and tables.

‡ Data were analysed by the χ^2 test or Fisher's exact test when appropriate. When looking for class II associations other than the well-established DRw17,DQw2 association^{5,7,8}, gene frequencies of patients and controls were compared after subtraction of DRw17,DQw2 positive haplotypes. *P* values were corrected (*P_c*) for multiple comparisons.

TABLE 2 Comparison of HLA-DQ β chain first domain polymorphic amino-acid residues in IgA-D-associated DQ alleles

Dw	DR	DQ	DQB1	IgA-D-assoc.	10	20	30	40	50	60	70	80	90
3	w17	w2	*0201	positive	RDSPEDFYVQ	FKGMCYFTNG	TERVRLVSRS	IYNREEIVRF	DSDVGEFRAV	TLGLPAAEY	WNSQKDILER	KRAAVDRVCR	HNYQLRLRTT
17	7	w2	*0201	positive	-----	-----	-----	-----	-----	-----	-----	-----	-----
1	1	w5	*0501	positive	-----	--L-----	-----G-T-H	-----Y--	-----VY--	-PQ-R-V--	-----EV--G	A--S-----	---EVAY-GI
2	w15	w6	*0602	negative	-----F-	-----	-----T-Y	-----YA--	-----VY--	-PQ-R-D--	-----EV--G	T--EL-T--	---EVAF-GI
		w7	*0301	neutral-neg.†	-----	--A-----	-----Y-T-Y	-----YA--	-----EVY--	-PL-P-D--	-----EV--	T--EL-T--	-----
8	w8	w4	*0402	neutral-neg.†	-----F-	-----	-----G-T-Y	-----YA--	-----VY--	-P--RLD--	-----E	D--S-T--	-----
18	w13	w6	*0603	neutral-neg.†	-----	-----	-----T-H	-----YA--	-----VY--	-PQ-R-D--	-----EV--G	T--EL-T--	-----

Numbers above the sequences refer to the amino-acid position. The protein sequences are given in standard amino-acid single-letter code and aligned to the DQ β allele of the DRw17,DQw2 haplotype. A dash indicates identity with this sequence. Position 57, bold. The DQB1 sequences are from the following sources: *0201 from refs 18, 19; *0501 from ref. 20; *0602 from ref. 26; *0301, *0402 and *0603 from refs 1, 2.

† *P* < 0.05 only before correction for multiple comparisons.

DQB1 alleles with negative and neutral-negative associations with IgA-D all have alanine at position 38 and threonine at position 77 (Table 2). As the DQB1 alleles *0302 and *0604 with neutral to positive associations share these residues^{1,2}, we consider it is less likely that these positions are primarily involved in the non-susceptibility to IgA-D.

Position 57 of the HLA-DQ β chain has been shown to be the most informative genetic marker for insulin-dependent diabetes mellitus (IDDM) in Caucasians and blacks so far identified^{1,2,12}. In IDDM Asp 57 confers resistance to the disease, whereas in the DRw6-associated subgroup of pemphigus vulgaris patients, Asp 57 is associated with disease susceptibility³. In a recent large population study of Norwegian IDDM patients¹³ roughly the same frequencies of non-Asp 57 (82%) and Asp 57 homozygous patients (2%) were found as in the present study of IgA-D patients. This is intriguing, as the associations with serologically defined HLA class II specificities partly differ in the two diseases. A 10-fold increase in the prevalence of IgA-D has been described in IDDM¹⁴. Furthermore, a higher incidence of IDDM in IgA-D has been observed in a small series of patients⁴. The increased co-occurrence of the two diseases might possibly be attributed to the shared HLA-DQ β 57 association.

In IgA-D, as in IDDM, the HLA class II association cannot be explained merely by the charge of the amino acid at position 57 (refs 1, 2, 15-17). It is tempting to speculate, that the class II association(s) might be influenced by other gene products of the HLA region acting as minor histocompatibility antigens. In IgA-D the primary association in the DRw17,DQw2 and DR7,DQw2 haplotypes might be with the HLA-DQ β chain, as this is identical in the two haplotypes^{18,19}. However, the sequences of the DQB1 gene in DR1,DQw5 (positively associated with IgA-D) and DRw10,DQw5 (with a neutral association) are identical^{20,21}. Thus, the DQB1 allele of the DR1,DQw5 haplotype most probably interacts with an allele of another locus, which may be DQA1 or DRB1. Furthermore, the protection conferred by the DRw15,DQw6 haplotype is more

pronounced than the non-susceptibility of other Asp 57 positive DQ β alleles. Notwithstanding these exceptions, the data indicating that residue 57 might be important in conferring susceptibility and resistance to IgA-D are striking. These findings favour the hypothesis that the HLA class II molecules themselves might be involved in the pathogenesis of IgA-D. □

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Sequence homology shared by neurofibromatosis type-1 gene and *IRA-1* and *IRA-2* negative regulators of the *RAS* cyclic AMP pathway

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NEUROFIBROMATOSIS type-1 (NF-1) is one of the most frequently inherited genetic disorders affecting humans¹. NF-1 primarily affects cells of neural crest origin and is characterized by patches of skin pigmentation (*café-au-lait* spots) and neurofibromas². Cloning of the human *NF-1* gene shows that it encodes an 11–13 kilobase transcript that is frequently disrupted in NF-1 patients^{3–5}. The frequent disruption of the *NF-1* gene in NF-1 patients combined with the autosomal dominant mode of inheritance of NF-1 strongly suggest that the *NF-1* gene is a tumour-suppressor gene. We have now sequenced a portion of the murine *NF-1* gene and show that the predicted amino-acid sequence is nearly the same as the corresponding region of the human *NF-1* gene product. Northern blotting identified mouse *NF-1* transcripts that are equivalent in size and complexity to those in human tissues, and Southern blotting shows that this region of the *NF-1* gene is evolutionarily well conserved. Finally, computer searches identified homology between the mouse *NF-1* gene and *IRA-1* and *IRA-2*, two genes identified in *Saccharomyces cerevisiae* that negatively regulate the *RAS*-cyclic AMP pathway. These findings provide important new insights into the possible function of the *NF-1* gene.

While cloning the human *NF-1* gene, we previously identified a complementary DNA clone, pmDV1, for the mouse *NF-1* gene. This clone contained a ~1.9-kilobase (kb) cDNA that we then used to identify cDNA clones for the human *NF-1* gene³. We determined the sequence of this 1.9-kb mouse cDNA (Fig. 1). The DNA sequence identifies a long open reading frame of 1,858 nucleotides. This sequence is completely contained in the sequence of the human *NF-1* gene⁴. Comparison of the mouse and human nucleotide sequences indicates that the two sequences are 91% identical. Among the 619 amino acids that can be compared between the two species, only three conservative amino-acid substitutions were observed (99.2% identity) (Fig. 1). These results indicate that this region of the mouse and human genes is highly conserved.

A 1.66-kb mouse NF1 probe generated by the polymerase chain reaction (PCR) (see Fig. 2 legend) was used in northern blots to identify transcripts from the murine *NF-1* locus (Fig. 2). In 4-day-old mice, an intense band of hybridization was detected in head RNA, faint hybridization detected in skin RNA, and no hybridization detected in body RNA (Fig. 2, lanes c–a, respectively). The size of this transcript is within the range of 11–13-kb reported for human *NF-1* transcripts.

In 14-day-old animals, *NF-1* transcripts were present in all tissues examined (Fig. 2, lanes d–g). Ubiquitous NF-1 expression also occurs in human tissues⁵. *NF-1* transcripts were also observed in the mouse B16 melanoma cell line (Fig. 2, lane h). In addition to the 11–13-kb transcript, two other transcripts were observed in melanoma RNA that were not detected in other tissues. It is not so far known whether these additional transcripts represent differential RNA splicing, different usage

of 5' or 3' ends, or transcripts from a related gene. In summary, the complexity and size of *NF-1* transcripts identified in mice were similar to those observed in humans.

The evolutionary conservation of the *NF-1* gene was further explored by hybridizing the 1.66-kb mouse PCR *NF-1* probe to genomic DNA, obtained from a number of divergent species, that had been digested with restriction endonuclease (Fig. 3). At both moderate stringency (Fig. 3, top panel) and high stringency (Fig. 3, bottom panel) washes, bands of hybridization were readily detected in DNAs of all species, including species as evolutionarily distant as *Xenopus laevis* (Fig. 3, lane i). These results provide further evidence that the *NF-1* gene has been highly conserved during evolution and indicates that this region of the *NF-1* gene is functionally important.

For insight into the function of the *NF-1* gene, we tried to identify genes that share amino-acid sequence homology with the mouse *NF-1* gene product by searching protein computer databases (Fig. 4 legend). Two search programs, FASTA and SEQFP, identified amino-acid sequence homology between NF-1 and the *S. cerevisiae* *IRA-1* gene product (inhibitory regulator of the *RAS*-cAMP pathway⁶). By using SEQFP, we show that three non-overlapping 100-amino-acid regions of NF-1 have sequence homology with *IRA-1*, indicating that the region of homology with *IRA-1* extends over a large region of the NF-1 protein. The amino-acid sequence comparison of mouse NF-1 and *IRA-1* is shown in Fig. 4. The two genes have 20.9% amino-acid identity (42.8% amino-acid homology).

The statistical significance of the amino-acid homology was examined using the program RDF⁷. RDF determines the significance of similarity, *z*, between two sequences (see Fig. 4 legend). For *z* > 6 (initial or optimal score), the homology between the two sequences is probably significant, and for *z* > 10 (initial or optimal score), the homology is considered to be significant. We obtained an initial *z* score of 7.5 and an optimal *z* score of 19.2 for NF-1 and *IRA-1*, indicating that the amino-acid sequence homology between the two gene products is highly significant.

The sequence of an *IRA-1*-related gene, *IRA-2*, has been published⁸. *IRA-1* and *IRA-2* have 45% amino-acid identity. The amino-acid sequence of *IRA-2* has not so far been incorporated into any of the genetic databases. Amino-acid sequence comparisons of *IRA-2* and mouse NF-1 again identified sequence homology between the two gene products (data not shown). *IRA-2* and NF-1 have 20.3% amino-acid identity (41.5% amino-acid similarity). The initial *z* score for NF-1 and *IRA-2* proteins was 13.9 and the optimal *z* score was 17.5, indicating that the amino-acid sequence homology between these two genes is also highly significant.

Like the *NF-1* gene, *IRA-1* and *IRA-2* genes encode large proteins^{6–8} of 2,938 amino acids and 3,079 amino acids, respectively. Both genes are transcribed as 10-kb messenger RNAs. Analysis of disruption mutants of *IRA-1* and *IRA-2* indicates that both genes act additively to negatively regulate the *RAS*-cAMP pathway⁸. In *S. cerevisiae*, disruption mutants of either *IRA-1* or *IRA-2* are viable but result in increased sensitivity to heat-shock and nitrogen starvation, and are defective in sporulation.

Ras proteins are involved in the control of proliferation and differentiation in mammalian cells^{9–10}. The activity of Ras proteins is modulated by their ability to bind and hydrolyse guanine nucleotides. GTP-binding activates Ras whereas GTP hydrolysis inactivates Ras. Mutant forms of Ras found in human tumours have greatly decreased GTPase activity, resulting in accumulation of Ras in the GTP-bound active form. Ras activity is normally regulated by accessory proteins that alter its ability to bind and hydrolyse GTP. For example, mammalian cells possess GTPase-activating proteins (GAP proteins) that catalytically accelerate hydrolysis of GTP bound to Ras protein¹¹.

In *S. cerevisiae*, RAS proteins control the activity of adenyl cyclase that produces the second messenger, cAMP. RAS

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1	TAT ATG ACC CCA TGG CTG TCA AAT CTA GTC CGT TTT TGT AAG CAT AAT	48	961	ATC CGA ATT CTT AGC AAG GCA CTT GAA AGT TGT TTA AAA GGA CCT GAC	1008
1	Y M T P W L S N L V R F C K H N	16	321	I R I L S K A L E S C T L K G P D	336
49	CAT GAT GCC AAA CGA CAA AGG GTT ACT GCC ATC CTT GAT AAG CTA ATA	96	1009	ACT TAC AAC AGT CAA GTT CTG ATA GAA TCT ACG GTG ATA GCA CTA ACA	1056
17	D D A K R Q R V T A I L D K L I	32	337	T Y N S Q V L I E S T V I A L T	352
97	ACA ATG ACT ATA AAC GAG AAG CAG ATG TAT CCT TCT ATT CAA GCA AAA	144	1057	AAA TTA CAG CCG CTT CTT AAT AAG GAC TCG CCC CTG CAC AAA GCC CTC	1104
33	T M T I N E K Q M Y P S I Q A K	48	353	K L Q P L L N K D S P L H K A L	368
145	ATC TGG GGG AGC CTT GGG CAG ATC ACA GAC CTG CTG GAT GTT GTG CTT	192	1105	TTT TGG GTT GCT GTG GCT GTG CTG CAG CTG GAC GAA GTC AAC TTG TAT	1152
49	I W G S L G Q I T D L L D V V L	64	369	F W V A V A V L Q L D E V N L Y	384
193	GAC AGT TTC ATC AAG ACC AGT GCG ACA GGA GGC TTA GGG TCT ATC AAA	240	1153	TCA GCC GGC ACT GCA CTT CTG GAA CAA AAC CTG CAC ACC TTC GAC AGT	1200
65	D S F I K T S A T G G L G S I K	80	385	S A G T A L L E S C L H T L D S	400
241	GCT GAG GTG ATG GCA GAC ACA GCT GTG GCT TTA GCT TCT GGA AAT GTG	288	1201	CTC CGG ATA TTC AAT GAC AAG AGT CCG GAA GAA GTA TTT ATG CGG ATC	1248
81	A E V M A D T A V A L A S G N V	96	401	L R I F N D K S P E E V F M A I	416
289	AAA TTG GTG TCG AGT AAG GTT ATT GGA AGG ATG TGT AAA ATA ATT GAC	336	1249	CGG AAC CCT CTG GAA TGG CAG TGC AAG CAG ATG GAT CAC TTT GTC GGA	1296
97	K L V S S K V I G R M C K I I D	112	417	R N P L E W H C K Q M D H F V G	432
337	AAG ACT TGC TTA TCC ACA ACT CCA ACT TTA GAA CAG CAT CTT ATG TGG	384	1297	CTC AAC TTC AAC TCT AAC TTT AAC TTT GCA CTA GTT GGA CAC CTC TTA	1344
113	K T C L S P T P T L E A G H L L	128	433	L N F N S N F A L V G H L L	448
385	GAC GAC ATT GCC ATT TTA GCC GCG TAC ATG CTG ATG CTG TCC TTC AAC	432	1345	AAA GGG TAC AGG CAT CCT TCA CCT GCC ATT GTT GCA AGA ACA GTC AGA	1392
129	D D I A I L A R Y M L M L S F N	144	449	K G Y R H P S P A I V A R T V R	464
433	AAC TCC CTC GAT GTG GCG GCT CAT CTG CCC TAT CTC TTC CAT GTT GTC	480	1393	ATT TTG CAT ACA CTA CTA ACT CTC GTT AAC AAA CAT AAT TGT GAC	1440
145	N S L D V A A H L P Y L F H V V	160	465	I L H T L L T L V N C K H R N C D	480
481	ACT TTC TTA GTA GCC ACA GGT CCC TTG TCC CTC CGA GCT TCC ACA CAT	528	1441	AAA TTT GAG GTG AAT ACA CAG AGT GTG GCT TAC TTA GCA GCT CTA CTC	1488
161	T F L V A T G P L S L R A S T H	176	481	K F E V N T Q S V A Y L A A L I	496
529	GGG CTG CTC ATC AAT ATC ATT CAC TCT CTG TGT ACT TGT TCT CAG CTT	576	1489	ACA GTG TCT GAA GAG GTT CGA AGT CCG TGT AGC CTC AAG CAT AGG AAG	1536
177	G L L I N I H S G A T C T C S Q L	192	497	T V S E V R S C K H R K	512
577	CAC TTT AGT GAA GAG ACC AAG CAA GTT TTG AGG CTC AGT CTA ACA GAG	624	1537	TCC CTT CTT CTT ACT GAT ATT TCA ATG GAA AAT GTT CCT ATG GAT ACA	1584
193	H F S E E T K Q V L R L S L T E	208	513	S L L L T D I S M E N V P M D T	528
625	TTC TCG TTA CCC AAA TTT TAC TTA CTG TTT GGC ATT AGC AAA GTC AAG	672	1585	ATT CAT CAT CAG GGT GAC CCC AGC TAT AAG ACA CTA AAG GAG ACC	1632
209	F S L P K F Y L L F G I S K V K	224	529	Y P I H H G D P S Y R T L K E T	544
673	TCG GCT GCT GTC ATT GCC TTC CGT TCC AGT TAC CGG GAC GCG TCC TTC	720	1633	CAG CCA TGG TCC TCT CCC AAA GGT TCA GAA GGA TAC CTT GCA GCC ACC	1680
225	S A A V I A F R S S Y R D R S F	240	545	Q P W S S P K G S E G Y L A A T	560
721	TCC CCT GGC TCC TAT GAG AGG GAG ACT TTT GCT TTG ACG TCC CTG GAA	768	1681	TAC CCA GCT GTA GGC CAA ACC AGT CCC CGA GGC AGG AAA TCC ATG AGC	1728
241	S P G S Y E R E T F A L T S L E	256	561	Y P A V G Q I S P R A R K S M S	576
769	ACA GTC ACA GAA GCT TTG TTG GAG ATC ATG GAG GCA TGT ATG AGA GAT	816	1729	TTG GAT ATG GGA CAG CCT TCT CAG GCC ACA AAA AAA TTG CTT GGA	1776
257	T V T E A L L E I M E A C M R D	272	577	L D M G Q P S Q A N T K K L L G	592
817	ATT CCA ACA TGC AAG TGG CTG ATG TGG ACA GAA CTA GCT CAA AGA	864	1777	ACA AGA AAA AGT TTT GAT CAC TTG ATA TCT GAC ACA AAG GCT CCT AAA	1824
273	I P T C K W L D Q W T E L A Q R	288	593	T R K S F D H L I S D T K A P K	608
865	TTT GCG TTT CAG TAT AAC CCA TCG CTG CAG CCA AGA GCT CTT GTG GTG	912	1825	AGA CAA GAA ATG GAA TCA GGG ATT ACA ACA CCC C	1858
289	F A F Q Y N P S L Q P R A L V V	304	609	R Q E M E S G I T T P	619
913	TTT GGC TGT ATT AGC AAA CGA GTG TCT CAT GGG CAG ATA AAG CAG ATT	960			
305	F G C I S K R V S H G Q I K Q I	320			

FIG. 1 Sequence of the murine *NF-1* cDNA clone. The 1,858 nucleotides of the pmDV1 cDNA clone that corresponds to the mouse *NF-1* gene are shown with the predicted product (single-letter amino-acid code). The numbers correspond to the nucleotide (upper line in each pair) or amino acid (lower line in each pair). The three amino-acid differences between the predicted murine amino-acid sequence and the predicted human amino-acid sequence are shown by the shaded boxes; the amino-acid differences occur at positions 179, 346 and 563.

proteins in *S. cerevisiae*, like those in mammalian cells, are regulated by several accessory proteins. The CDC25 protein positively regulates RAS function by catalysing the exchange of GDP bound to RAS for GTP¹²⁻¹⁵. By contrast, IRA-1 and IRA-2 proteins suppress the activity of RAS by GTP hydrolysis of RAS-GTP. The phenotype of yeast cells that have lost this negative regulatory function resembles that of yeast cells carrying the constitutively active form of RAS found in human

METHODS. The mouse *NF-1* cDNA clone (pmDV1) was isolated from a mouse macrophage cDNA library³ and was sequenced by the dideoxy chain termination method¹⁸ using a Sequenase kit (USB) and double-stranded plasmid DNA. Sequencing primers were either the T3 or T7 polymerase oligonucleotide primers or synthetic oligonucleotides from previously determined sequences. The 5' end of the plasmid insert had a run of 72 T residues flanked on either end by *EcoRI* linkers; this sequence is presumably a cloning artefact, as it is not represented in the human cDNA clones⁴.

tumours. Both IRA-1 and IRA-2 share limited but significant amino-acid sequence homology with mammalian GAP, and mammalian GAP can suppress the phenotype of *ira* mutants¹⁶. This suggests that IRA-1 and IRA-2 may be functionally equivalent to mammalian GAP.

The GAP homology domain of IRA-1 and IRA-2 has been localized to a small region in the middle of both proteins in amino acids 1,451-1,780 (IRA-1) and 1,597-1,925 (IRA-2)¹⁶.

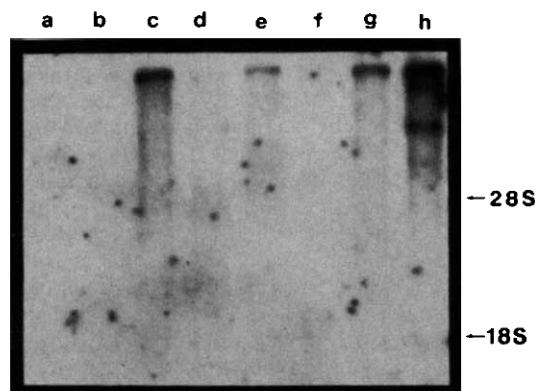


FIG. 2 Expression of murine *NF-1* mRNA. Poly(A)⁺ RNA (twice selected) was examined by northern blotting using the mouse *NF-1* PCR probe. The RNA (5 µg per lane) was from body (a), skin (b) or head (c) of 4-day-old DBA/2J mice, or spleen and thymus (d), kidney (e), liver (f), or brain (g) of 14-day-old DBA/2J mice, and B16 melanoma cell line (h). The position of migration of 28S and 18S RNA is shown. Less RNA was loaded in lanes a and b than in the other lanes, as determined by hybridization with a β-actin control probe (data not shown).

METHODS. Total RNA was isolated from tissues using the LiCl-urea method¹⁹. Poly(A)⁺ twice-selected RNA²⁰ was size-fractionated on 1.0% agarose-formaldehyde gels²¹. The DNA probe used for both Southern and northern blot hybridization was PCR-generated using the mouse *NF-1* cDNA clone pmDV1³ as a template. The amplimers (CATAATGATGATGCCAACGA and GACTGGTTTGGCCTACAGCT) used to generate the probe were derived from the known *NF-1* DNA sequence and the resulting 1.66-kb probe was purified on an agarose gel and was devoid of plasmid sequences. Northern blots were hybridized and washed as described²².

The small size of the essential GAP domain and the large size of the IRA proteins suggests that sequences flanking the GAP domain may have important regulatory functions. For example, IRA proteins seem to function as detectors of nutritional limitation. Therefore, one role for these flanking sequences may be to monitor the level of nutrients in the external environment and transduce that information to the GAP catalytic domain, which, in turn, effects the level of GTP bound to RAS.

The amino-acid sequence homology we have observed between the *NF-1* and *IRA* gene products, combined with the unusually large size of each transcript, raises the possibility that the *NF-1* and *IRA* genes have a common ancestor and that *NF-1* functions as a negative regulator of signal transduction in mammalian cells in a manner analogous to the *IRA* genes in *S. cerevisiae*. Such a mode of action of the *NF-1* gene would explain the basis for the uncontrolled growth of neural crest-derived cells seen in patients with mutations in the *NF-1* gene and would also be consistent with a tumour-suppressor gene, which is the postulated mode of action of the *NF-1* gene. Additional sequencing studies of the *NF-1* gene coupled with the development of biological assays for the *NF-1* gene will be necessary to confirm this possibility. The homology we have detected between *NF-1* and *IRA* is in the C terminus of each protein^{4,5}. As additional segments of the *NF-1* gene are cloned

and sequenced, it will be interesting to see if the homology we have detected between *NF-1* and *IRA* extends into the N-terminal and GAP domains of the *IRA* proteins.

The strong evolutionary conservation of the *NF-1* gene suggests that model organisms such as the mouse will be useful for developing assays for elucidating the functions of the *NF-1* gene. For example, mutations can now be introduced into the mouse *NF-1* gene using techniques for homologous recombination in mouse embryonic stem cells. Not only will this approach shed further light into the functions of the *NF-1* gene, but it may also produce a useful mouse model for the study *NF-1* disease.

The identification of two related *IRA* genes in yeast raises the possibility that there are many *IRA*-related genes in

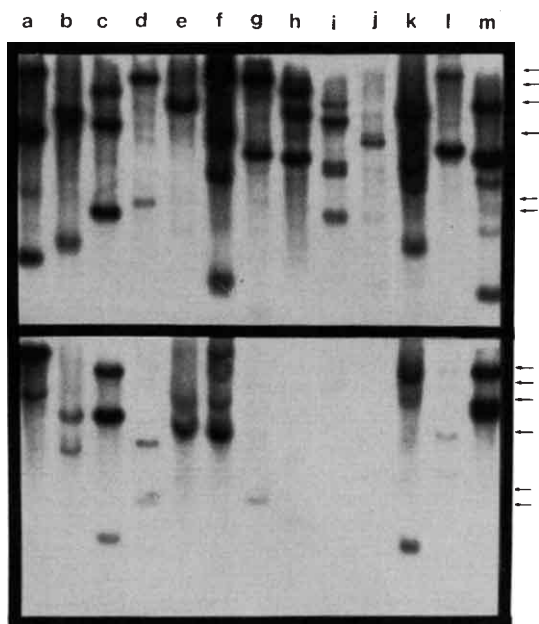


FIG. 3 The *NF-1* gene has been well-conserved throughout evolution. Genomic DNAs from a variety of different species were cleaved with either *EcoRI* (upper panel) or *HindIII* (lower panel) and examined for sequences that crosshybridize to the mouse *NF-1* PCR probe on Southern blotting. The hybridization was performed in $4\times$ SSCP at 55 °C for 16 h with a final wash in either $1\times$ SSCP 55 °C (upper panel) or $0.2\times$ SSCP 65 °C (lower panel). The DNA (10 µg per lane) was isolated from hamster (a), guinea-pig (b), rat (c), dog (d), cat (f), chicken (g), turkey (h), *Xenopus laevis* (i), rabbit (j), sheep (k), human (l) or C57BL/6J mice (m). The arrows indicate the mobility of *HindIII*-cleaved lambda DNA electrophoresed in parallel lanes of the same gels and correspond to 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb (from top to bottom).

METHODS. High-relative molecular mass DNA was prepared from frozen tissue samples as described²³. Restriction endonuclease digestions, agarose gel electrophoresis and Southern blot transfers were performed as described²⁴. Hybridization was at 55 °C in $4\times$ SSCP, 0.1% SDS, 10% dextran sulphate, $4\times$ Denhardt's solution and 0.1 mg ml⁻¹ salmon sperm DNA. Washes were as follows: three times in $1\times$ SSCP, 0.1% SDS at 55 °C, for 30 min each (*EcoRI* Southern blot); and for the *HindIII* Southern blot, additional washes of $0.5\times$ SSCP, 0.1% SDS at 65 °C and $0.2\times$ SSCP, 0.1% SDS at 65 °C were performed for 30 min each.

```

1 .....YMTPLSNLV.RFCKHNDQAKRQRTAILDKLITMTINEKQMP 43
2270 IPTVCSLSYWPVNLVYEHVLANDEEGPEATSRITYSLIRLTVPKPNFTT 2319
44 SIQAKIWGLGQITDLDVLSFKTSATGGLSGIKAEVMDATAVALAS 93
2320 AYLQQTWFLALDGRITNVIIEIV..SHALDRDSNRDWMKAVST..LTS 2366
94 GNVKLVSSKVIKGMCKIIDTKLSPPTLQELHMLDDIAILARYMLMSF 143
2367 FPTTEIACQVIEKLINMI.KSFLPSLAVERSAHSWELTILSKISVSIF 2415
144 NNSLDVAALPYLPHVTVFLVATGSLRSTHGLLINIHSCTCSCQLH 193
2416 ESPLSQMYLPEILFAVSLTIDVGPSEIRVSLYELLMLNVCHSLTNESL 2464
194 FSEETKQVRLSLTEFLPKFYLLFGISKVKSAAVIAFRSSYDRSRFSPG 243
2465 .PERNRKNDIVCATFARQKLNFSIGFSQEGKRVLPNFAAS...SFSS. 2508
244 SYERETFALTSLETVEALLEIMEACMRDITPCKWLDQWTELAQRFQY 293
2509 .....KFGTDLDFTKNIMLME..YGSISEGAQWEAKYKYLMDAIFGH 2550
294 NPSLQPRALVFGCISKRVSHGQIKQIIRILSKALESCLKGPDTYNSQYL 343
2551 RSFFSARAMMILGIMSK..SHTSLFLCKELLVETMKVFAEPVVDQEMFI 2598
344 IESTVIALTKLQPLLNKDSPLHKAFLVAVAVLQLEVNLSAGTALLEQ 393
2599 IIAHVFTYSKIVEGLDPSSELMKELFWLATIJCVEPHLLFEGGLFMVN 2648
394 NLHTLDSLR...FNOKSPEEVFMAIRNPLEWHCKQMDHFVGLNFN.SNF 439
2649 CLKRLYTVHLQGLGFGKSLAKLMESRNFATLLAKLESYNGCIWNEDF 2698
440 NFALVGHLLKGYRHPSPAIVARTVRILHTLT..... 471
2699 PHILGFIANG..SIPVVKGAALDCLQALFKNTYERKSNPKSSDYLCY 2746
472 ..LVNKHNRCDKFEVNTQSVAYLAALLTVSEEVSRCSLKHRS..... 513
2747 LFLHLVLSPEQLSTLLLEVGFEDELVPLNNTLVPLTLINWSSDSKKS 2796
514 .....LLLTDSIMENVP.....MDTYPPIHGDPSYRILKET 544
2797 NIVLYQGALLFSCVMSDEPCKFRFALLMRYLLKNPFCVFRYTLTRKEF 2846
545 QWSSPKGSEGYLEATYPVAGQTSPRARKMSLMD.GQPSQANTKKLLGT 593
2847 RRLSTLEQSEAVASFELIGMLVTHSEFNYLEEFNDEMVELLKKRGLSV 2896
594 RKSFDHLISDTKAPKQEMESGITPRN..... 621
2897 VKPLDIFDQEHIEKLGEGEHQVAIERKRLATMILARMSCS 2938

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FIG. 4 Amino-acid (single-letter code) sequence comparison of mouse *NF-1* and yeast *IRA-1*. Alignment of the known predicted amino-acid sequence of mouse *NF-1* (top sequence) with the C-terminal region of *IRA-1* (lower sequence). Identical amino acids are shown by the solid vertical line, conservative amino-acid substitutions are shown by the colons (these amino acids are grouped as ILMV, KHR, DENQ, STPAG and FWY; single-letter code). Alignment was performed using the GAP program in the University of Wisconsin Genetics Computer Group package²⁵. The periods indicate the placement of gaps to maximize alignment between the sequences. **METHODS.** Sequences were analysed using the software package of the Genetics Computer Group²⁵. Homology to *IRA-1* and *IRA-2* was identified using both the FASTA program²⁶ and the SEQFP program in the IDEAS package²⁷. The Genbank (release 64.0), NBRF (release 25.0), EMBL (release 22.0) and SwissProt (release 14.0) databases were screened for homologous sequences. SEQFP and FASTA are based on the same algorithm⁷. Alignment scores (*z* values) were determined using the program RDP⁷. RDP determines both the initial (without introduction of gaps) and optimal (after allowing for insertions and deletions) scores of a sequence alignment and then randomizes one of the sequences many times (100) and determines the mean of the random scores. The *z* values were determined using $k_{\text{top}}=2$ and $N=100$.

mammals. Indeed, a second form of neurofibromatosis has been identified, neurofibromatosis type-2 (NF-2). Mapping studies indicate that the *NF-2* locus, in at least some families, is located on human chromosome 22 (ref. 17). Once the *NF-2* gene is cloned it will be important to determine whether it too shares sequence homology with *IRA*-related genes. □

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Frequent activation of N-myc genes by hepadnavirus insertion in woodchuck liver tumours

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THE recent finding of c-myc activation by insertion of woodchuck hepatitis virus DNA in two independent hepatocellular carcinoma¹ has given support to the hypothesis that integration of hepatitis B viruses into the host genome, observed in most human and woodchuck liver tumours^{2,3}, might contribute to oncogenesis. We report here high frequency of woodchuck hepatitis virus DNA integrations in two newly identified N-myc genes: N-myc1, the homologue of known mammalian N-myc genes, and N-myc2, an intronless 'complementary DNA gene' or 'retropon' that has retained extensive coding and transforming homology with N-myc. N-myc2 is totally silent in normal liver, but is overexpressed

without genetic rearrangements in most liver tumours. Moreover, viral integrations occur within either N-myc1 or N-myc2 in about 20% of the tumours, giving rise to chimaeric messenger RNAs in which the 3' untranslated region of N-myc was replaced by woodchuck hepatitis virus sequences encompassing the viral enhancer. Insertion sites were clustered in a short sequence of the third exon that coincides with a retroviral integration hotspot within the murine N-myc gene, recently described in T-cell lymphomas induced by murine leukaemia virus^{4–6}. Thus, comparable mechanisms, leading to deregulated expression of N-myc genes, may operate in the development of tumours induced either by hepatitis virus or by nonacute retroviruses in rodents. Activation of myc genes by insertion of hepadnavirus DNA now emerges as a common event in the genesis of woodchuck hepatocellular carcinoma.

We have cloned and sequenced a single woodchuck hepatitis virus (WHV) insertion site from an early hepatocellular carcinoma (HCC) carried by a chronically infected woodchuck (W204). The WHV insert, a subgenomic fragment encompassing the viral enhancer, is flanked by host sequences that are not contiguous in the normal woodchuck genome (Fig. 1c). We subcloned a 1.7-kilobase (kb) DNA fragment of the 5' host-viral junction (BA1700, Fig. 1c), showing homology with N-myc sequences and no repetitive elements, and used this to further screen the W204 HCC genomic library. This approach yielded two subsets of clones, derived from distinct genomic loci. The locus depicted in Fig. 1a was identified by nucleotide sequencing as the woodchuck homologue of the human and murine N-myc genes^{7–9}, similarly organized into three exons. It was thereafter designated as N-myc1. The second locus, termed N-myc2, represents the unoccupied allele of the 5' flanking cellular sequences in W204 HCC (Fig. 1b), and is part of the normal woodchuck genome, as shown by Southern analysis (Fig. 3).

Nucleotide sequencing of N-myc2 (Fig. 2a) revealed a structure typical of a cDNA gene or retroposon. The N-myc homology starts at the exact 5' end of the first exon⁸, extends through exon 1 to the first splice donor site¹⁰, then over the entire exons 2 and 3, showing no intervening sequences. It terminates at the 3' end of the third exon, 16 base pairs (bp) downstream of a consensus polyadenylation sequence, and is flanked at its 3' side by an adenosine-rich stretch of 53 nucleotides, which might constitute the remnants of a poly(A)⁺ tail. A sequence of 16 nucleotides is directly repeated at each end of the N-myc-like sequence (positions –325 and 2,241, Fig. 2a). These data indicate that N-myc2 may have evolved from retroposition of one of the two alternatively spliced N-myc mRNAs¹⁰. This process, or the subsequent absence of selective pressure, is usually associated with genetic lesions that prevent translation of any transcript into functional polypeptides¹¹. However, a N-myc-like open reading frame is preserved in the normal N-myc2 allele (Fig. 2), as well as in the mutated allele of W204 HCC, interrupted by WHV DNA insertion 6 bp downstream of the stop codon (Fig. 1c).

The predicted N-myc2 coding region encodes a polypeptide of 454 amino acids showing 80% and 78% homology with the woodchuck and human N-Myc proteins and extensive conservation of the functional domains of known Myc proteins (Fig. 2b). As in other N-myc genes, the coding region begins with three ATG codons, closely spaced and in-phase; the first and the second ATG triplets of the human N-myc gene encode the N-terminal methionine residues of two N-myc polypeptides¹². The stretch of acidic amino acids around position 270 of N-Myc2, common to c-Myc and N-Myc proteins, possibly coincides with a transcriptional activation region¹³ and/or with a protein instability motif¹⁴. The highest degree of homology between N-Myc2 and other N-Myc proteins is encountered in the carboxy terminal region, which contains a basic motif and an amphipathic helix-loop-helix bearing a leucine zipper, as indicated in Fig. 2b. This complex structure, common to Myc proteins and other regulatory factors, is believed to be critical for specific DNA-binding and protein dimerization

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functions^{15,16} and is important for the transforming activity of c-Myc¹⁷ and N-Myc¹⁸.

A characteristic functional property of mammalian *myc* genes is their ability to cooperate with an activated *ras* gene in tumorigenic conversion of primary embryo fibroblasts¹⁹⁻²¹. To assess whether N-*myc2* has retained this function, we cotransfected pRSV-N-Myc2 (a plasmid carrying the 2,627-bp *RsaI*-*HindIII* fragment of N-*myc2* cloned into the *HindIII* and *HpaI* sites of pRSVCAT) and pSV₂neo-*ras* (a c-Ha-*ras1* (EJ)²² expression vector) into rat embryo fibroblasts using standard conditions¹⁹. Neomycin-resistant foci of transformed cells were obtained with the same efficiency as in rat embryo fibroblasts transfected with pSV-c-myc-1²³ and pSV₂neo-*ras*. When foci carrying EJras together with either N-*myc2* or c-*myc* genes were picked, they yielded established lines of overtly transformed, rapidly growing cells (manuscript in preparation). Both types of cotransfected cells were consistently tumorigenic when injected into nude mice, producing tumours that reached diameters of 1-3 cm, two weeks after inoculation (results not shown). Thus, N-*myc2* can replace c-*myc* in rat embryo fibroblasts cotransformation and tumorigenicity assays.

A survey of 30 fresh, independent liver tumours, using Southern blot hybridization of *HindIII*-digested genomic DNA with a N-*myc* probe revealed DNA rearrangements of a N-*myc* allele in six additional tumours (Fig. 3a). In all cases except W97 HCC, the tumour-specific N-*myc* bands comigrated with viral bands (Fig. 3b), suggesting junction fragments of N-*myc* and integrated viral sequences as in W204 HCC. Remarkably, all rearranged *HindIII* fragments of individual tumours annealed to probe WHV En, a 600-bp subgenomic fragment encompassing the viral enhancer element²⁴ (Fig. 3b). Only in W134B HCC could the tumour-specific band also be revealed by a N-*myc1*-specific probe (Fig. 3c). Rearrangements of N-*myc2* were confirmed in the remaining six tumours, using *Bam*HI digests and the N-*myc2*-specific probes illustrated in Fig. 1 (data not shown). In addition, *Bam*HI or *TaqI* digestion

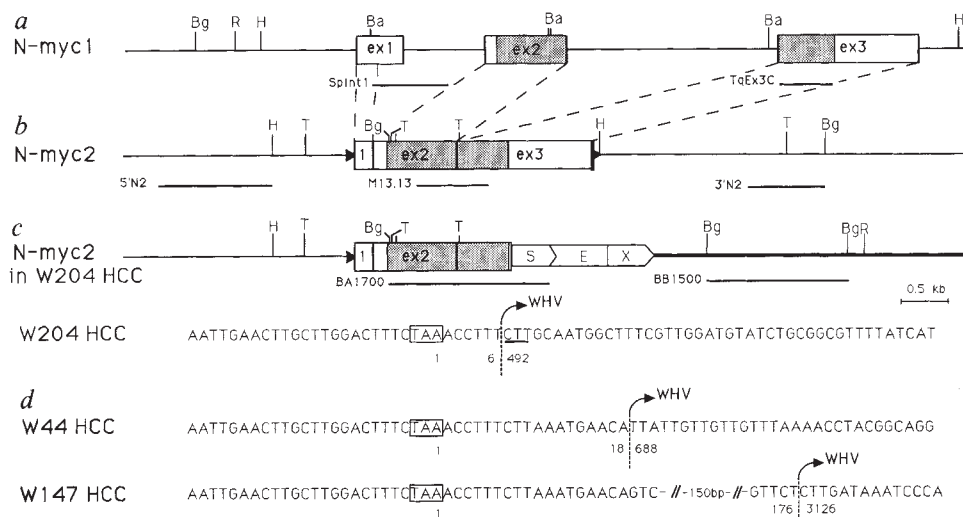
of tumour DNAs and hybridization with a probe of the N-*myc1* exon-3 coding region localized the integration sites in the third exon of N-*myc1* (in W134TB) or in the homologous region of N-*myc2* (in the remaining tumours) (Fig. 3d).

Elevated levels of N-*myc*-related transcripts ranging from 3-4.5 kb in size were detected in four woodchuck HCCs (W147, 44, 134B and 264) (Fig. 4a). Fainter bands at 3.4 and 4.2 kb in W204 HCC and at 3.5 kb in 1337 HCC were seen after longer exposures. Northern blot hybridization with probe WHV En identified viral transcripts of abnormal sizes, comigrating with N-*myc*-related transcripts in all six tumours (Fig. 4b), suggesting co-transcription of N-*myc* and viral sequences from the mutated alleles. This was confirmed in W44 and W147 HCCs by a polymerase chain reaction (PCR) procedure on cDNA made from total cellular RNA, using oligonucleotides located on each side of the putative host-viral junctions and sequencing of the amplification products (Fig. 1d). In both tumours, as in W204 HCC, integration of viral DNA directly downstream to the N-*myc2* coding region and in the same transcriptional orientation produced truncated N-*myc* RNAs in which the 3' untranslated region of N-*myc* was replaced by WHV sequences. The viral enhancer was retained in these transcripts, as shown by northern hybridization (Fig. 4b) and sequencing of cloned DNA fragments from the host-viral junctions (Fig. 1c, d). Furthermore, the host-viral junctions in the three tumours analysed are localized in different parts of the WHV pre-S/S region, but less than 200 bp apart within the third N-*myc2* exon. These sequences, previously identified as the unique target for retroviral insertion into the murine N-*myc* gene in murine leukaemia virus-induced T cell lymphomas⁴⁻⁶, might be highly recombinogenic for reasons not yet understood.

Several other woodchuck tumours produce abundant 2.3-kb N-*myc* transcripts in the absence of gross genetic alterations of the N-*myc* genes (Fig. 4a). These RNAs were not detected by a N-*myc1*-specific probe (Fig. 4c) and were identified as N-*myc2* transcripts by PCR experiments using cDNA made from total

FIG. 1 Structural comparison of N-*myc1*, N-*myc2*, and rearranged N-*myc2* alleles. a, Organization of N-*myc1*. Boxes, the exons with coding region shaded. Splnt1 is a N-*myc1*-specific probe, TqEx3C detects both N-*myc* genes. Ba, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; EcoRI; T, *Taq*I (not all sites are shown). b, Organization of the germline N-*myc2* allele. Regions of homology with N-*myc1* exons are aligned with dotted lines. The 3' poly(A) stretch is represented as a thick vertical bar and the flanking direct repeats as two black arrowheads. 5'N2 and 3'N2 are N-*myc2*-specific probes, M13.13 hybridizes with N-*myc1* and N-*myc2*. c, Restriction map of the occupied N-*myc2* allele in W204 HCC and sequence of the left host-virus DNA junction. Narrow open box, the 1.5 kb WHV insert indicating the viral (+) strand orientation. S and X, viral genes; E, enhancer element. Thin line, N-*myc2* locus; thick line, 3' flanking cellular sequences different from N-*myc2*. N-*myc2* sequences are numbered relative to the last base pair of the stop codon (boxed) and WHV sequences from ref. 34. The host-viral junction is indicated by an arrow and the underlined triplet CTT corresponds to junctional homology of viral and cellular DNA. d, Sequence analysis of the N-*myc2*-WHV junction in W44 HCC and W147 HCC chimaeric transcripts.

METHODS. An Emb3 recombinant phage library was constructed from size-fractionated DNA of W204 HCC partially digested by *Sau*3A and screened with a probe for WHV DNA¹. Restriction fragments from the viral-host junctions (BA1700, a 1.7-kb *Bgl*II-*Apal* fragment and BB1500, a 1.5-kb *Bgl*II fragment) were subcloned into Bluescript vectors (Stratagene). The N-*myc1* and N-*myc2* genomic clones were obtained by screening the same library with BA1700 as a probe. Restriction fragments from the N-*myc1* gene



(Splnt1, a 800-bp *Sal*I-*Sph*I fragment, and TqEx3C, a 450 bp *Taq*I fragment) and from the germline N-*myc2* allele (5'N2, a 1.2-kb *Sal*I-*Hind*III fragment and 3'N2, a 800-bp *Xba*I-*Bgl*II fragment) were cloned and used as probes. The nucleotide sequence of the N-*myc1* gene will be published elsewhere. The 3.5-kb *Hind*III fragment of the germline N-*myc2* allele and the 6.4-kb *Hind*III-*Eco*RI fragment of the viral insertion site were fully sequenced³⁵ using a shotgun strategy³⁶. M13.13, a 750-bp insert from a M13 clone, was subsequently subcloned into Bluescript. For analysis of the chimaeric transcripts from W44 and W147 HCCs, the reverse transcriptase-polymerase chain reactions were performed essentially as described³⁷, using as primers a WHV-specific oligonucleotide of the viral enhancer core (position 1,309-1,328) and the N-*myc2*-specific oligonucleotide OLN3 (see Fig. 2). Omission of the reverse transcriptase or testing normal liver RNA yielded parallel negative controls.

activated in liver tumours, either by downstream insertion of WHV or in the absence of genetic rearrangements. This cDNA gene stands presently as one of the few cases in which processed copies of intron-containing genes have evolved into functional genes^{26,27}. The intronless rat *s-myc* gene, which has lost the transforming capacity of the parental *myc* gene and acquired a

suppressive effect on tumorigenicity, is a seemingly related example²⁸. In this context, it will be interesting to determine whether *N-myc2* is expressed in the developing embryo or in adult woodchuck tissues, and whether it has acquired some specific function in normal cells. In addition, together with the activation of *c-myc* previously described¹, we report insertional

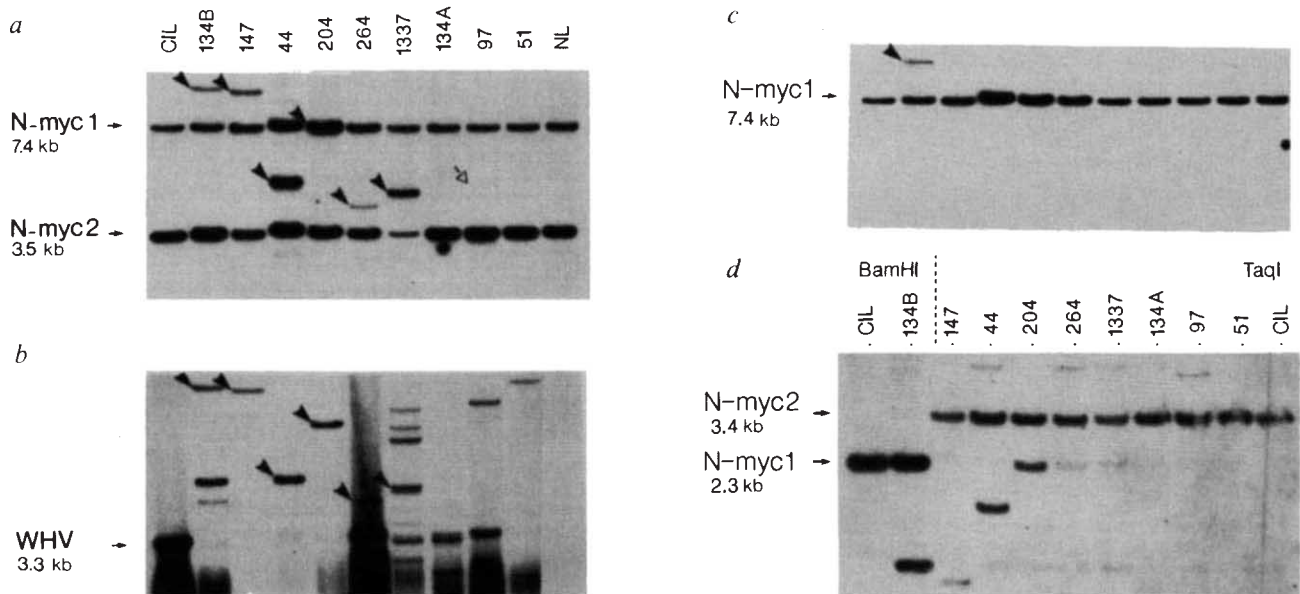


FIG. 3 Integration of WHV DNA into *N-myc* genes in woodchuck HCC. *a*, *b* and *c*, Southern blot analysis of *Hind*III-digested DNA from nine woodchuck HCCs (named above each lane), a WHV-infected liver bearing no HCC (CIL) and a normal liver (NL), hybridized sequentially with the *N-myc* probe M13.13 (*a*), the viral subgenomic fragment WHV En (*b*) and the *N-myc1*-specific probe Splnt1 (*c*). Solid arrowheads indicate tumour-specific DNA bands detected by the *N-myc* and WHV probes. Open arrow marks the W97 HCC-specific *N-myc2* band that failed to hybridize with the viral probe. Viral replicative forms are seen in (*b*) as smears beneath the indicated position of the double-stranded WHV genome. Fragments of higher relative molecular mass represent viral sequences integrated into the cellular genome. 134A and B were two different tumour nodules on the same woodchuck liver. *d*,

Southern blot showing DNA rearrangements in the third exon of either *N-myc1* (in W134B HCC) or *N-myc2* (in six other tumours). *N-myc2* bands that are fainter than the *N-myc1* bands reflect incomplete annealing of the *N-myc1* TqEx3C probe to *N-myc2* exon 3 sequences at high stringency. METHODS. Total DNA was prepared⁴ from frozen liver and tumour samples, digested with different restriction enzymes, separated by size (20 µg DNA per lane), blotted in alkali to Hybond N⁺ membranes (Amersham) and hybridized with randomly labelled probes as described³⁸. WHV En is a 612-bp *Apal*-*Nco*I fragment (position 888–1,500). After hybridization with TqEx3C, washings were performed at higher stringency (0.08 SSC buffer, 0.1% SDS, 65 °C) to minimize annealing of the probe to *myc* sequences different from *N-myc*. The filters were exposed at –80 °C overnight.

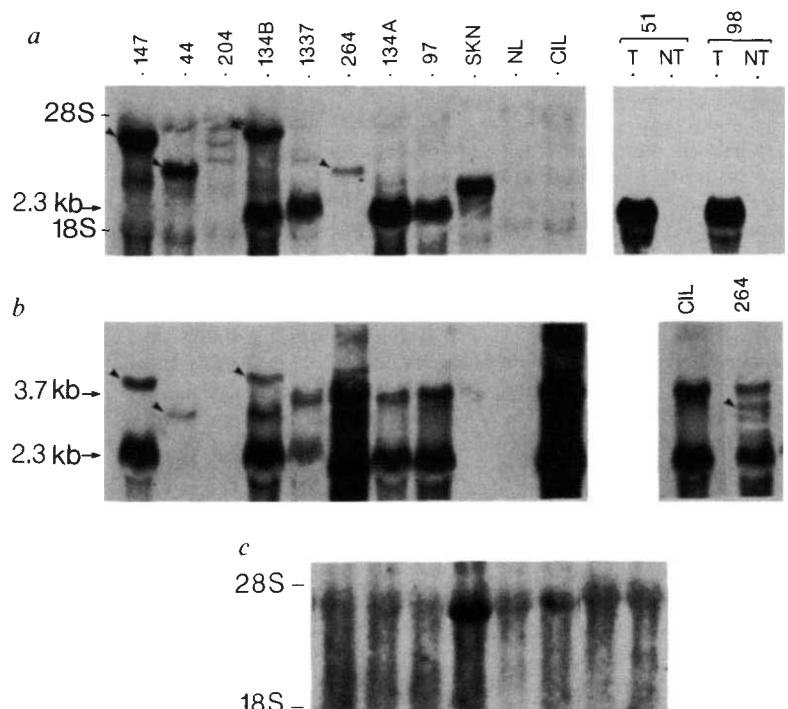


FIG. 4 Altered *N-myc* expression in woodchuck HCC. *a*, Northern blot of liver and tumour RNA, prepared from the biopsy samples also used for DNA analysis (Fig. 3), from the tumorous (T) and non-tumorous (NT) parts of two livers (W51 and W98) showing no rearrangements of *N-myc* genes (W98 HCC DNA not shown) and from the human neuroblastoma cell line SKN-B2. The M13.13 probe detects *N-myc1* and *N-myc2* transcripts. *b*, Same blot probed with WHV En. The sizes of the normal WHV transcripts are indicated. Shorter exposures are shown for W264 HCC and WHV-infected liver RNAs in the right lanes. *c*, Northern blot hybridization with the *N-myc1*-specific probe Splnt1. RNA samples were as in the first eight lanes of *a* and *b*. Only the 4.5-kb RNA in W134B HCC is detected. Positions of 28S and 18S ribosomal RNAs are indicated. METHODS. Total RNA was prepared and analysed as previously described²; 40 µg RNA was loaded per lane, except for SKN-B2 RNA (10 µg). The filters were exposed at –80 °C for 3 days (*N-myc* hybridization) or for 6 h (WHV hybridization).

mutagenesis of *myc* genes by WHV DNA in about one third of the hepatocellular carcinoma examined (10% c-*myc*; 20% N-*myc*). These results clearly establish that woodchuck hepatitis virus, like nonacute retroviruses, contributes to neoplastic transformation through insertional activation of cellular proto-oncogenes. WHV is closely related to the human hepatitis B virus (HBV) and serves as a model for liver disease and for hepatocarcinogenesis in humans. Woodchuck hepatomas, however, differ notably from a majority of human hepatomas in the earlier onset of tumours in the carrier state and in the absence of associated cirrhosis, suggesting that HBV might act more indirectly. Transactivation of cellular genes by the truncated products of integrated HBV preS2/S genes²⁹ and by the X protein³⁰, and long-term storage of the viral glycoprotein encoded by the pre-S1/pre-S2/S region³¹ have been proposed as causative factors in HBV-associated oncogenesis. HBV integration near *myc* genes or other currently known proto-oncogenes has not been observed thus far; however, the finding of HBV insertion sites within retinoic acid receptor β and cyclin A, two genes essential for cell growth and differentiation, in two independent tumours^{32,33}, supports the idea that HBV may act, at least occasionally, as an insertional mutagen. □

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Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor

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THE vitamin A derivative, retinoic acid, can regulate morphogenesis and differentiation in vertebrates^{1,2}. Two different subfamilies of the steroid receptor superfamily of transcription factors, the retinoic acid receptors and the retinoid X receptor, mediate the effects of retinoic acid³⁻⁶. As part of an analysis of the hormonal control of development, we have examined the *Drosophila* genome for retinoic acid receptor homologues⁷. Here we describe one such gene, *XR2C*, which encodes a product with structural similarity to the human retinoic acid-responsive transcription factor, retinoid X receptor. This receptor-like protein is encoded by *ultraspiracle* (*usp*), a locus required both maternally and zygotically for pattern formation. The discovery that the *usp* product is a retinoid X receptor homologue suggests that similar chemical cues underlie morphogenic signalling in vertebrate and invertebrate systems.

Potential homologues of vertebrate steroid hormone receptor-like genes were identified by probing a blot of wild-type *Drosophila* genomic DNA with DNA encoding the human retinoic acid receptor (hRAR α) DNA-binding domain. The genes corresponding to the four darkest bands were cloned,

sequenced and cytologically mapped⁸, but none of the isolates showed greater than 60% sequence identity to the RAR α in the DNA-binding domain. One class of isolates maps to cytologic position 2C on the X chromosome (not shown) and was called XR2C. A 300-base pair (bp) *Pst*I-*Bam*HI fragment (PB), encoding the XR2C putative DNA-binding domain (Fig. 1a), hybridizes to a single fragment in both *Eco*RI- and *Xho*I-digested genomic DNA (Fig. 1b), demonstrating that XR2C is a single-copy gene. Further, the PB probe recognizes a few cross-hybridizing bands on Southern blots of vertebrate genomic DNA (Fig. 1c), indicating that XR2C-related sequences are conserved throughout the vertebrate lineage. Although XR2C was isolated with hRAR α probe, the bands that cross-hybridize with RAR α gave a signal below detection in the human lane. Instead, the pattern of hybridization is the same as that seen for the human retinoic acid-responsive transcription factor, retinoid X receptor (RXR α) (see below)⁶.

The structure of the XR2C transcripts was determined from the sequence of complementary DNA clones isolated by screening larval and pupal cDNA libraries with the PB probe. The predicted protein product reveals a marked similarity with the hRXR. The nucleotide sequence of the longest insert is shown in Fig. 2a and a comparison of the predicted amino-acid sequence of XR2C with that of other nuclear receptors in both vertebrates and *Drosophila*, is shown in Fig. 2b, c. XR2C is 86% identical to RXR α in the DNA-binding domain but only 59% in the same region of the hRAR α . XR2C shares 49% identity in the hormone-binding domain with RXR and only 24% with the RAR. These results confirm the evolutionary conservation of the RXR subfamily.

Comparison of the XR2C restriction map to that of a previously published genomic walk of the 2C-2E region, oriented the XR2C transcript to the chromosome and to several key deletion breakpoints⁹. Genetic characterization of lethal mutations relative to these breakpoints identified three possible candidate genes for XR2C: *l(1)2Cc*, *l(1)2Ce* and *ultraspiracle* (*usp*)^{10,11}. To assign a locus to the XR2C gene, blots of genomic DNA from alleles of these genes were examined for associated rearrangements in the XR2C transcription unit.

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Only one mutant, *usp*^{KA21}, was associated with a rearrangement in *XR2C* (not shown). To localize the precise breakpoint relative to the *XR2C* coding region, a genomic library from heterozygous *usp*^{KA21} females was constructed and screened with the 8-kilobase (kb) *EcoRI* fragment that contains *XR2C* and spans the breakpoint. Analysis of the structure of clones

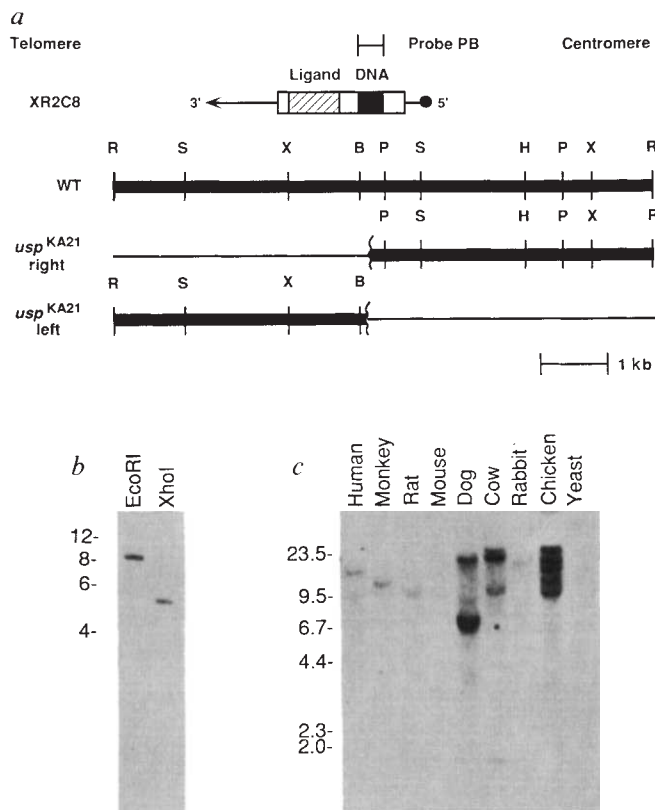


FIG. 1 Structure, copy number and evolutionary conservation of *XR2C*. a, Schematic diagram of the structure of the *XR2C* locus. A restriction map of the wild-type (WT) genomic region is shown as a thick line. The structure and extent of the transcription unit is indicated above the genomic map, as is the position of the *BamHI*-*PstI* fragment used as hybridization probe (probe PB). All of the cDNAs isolated were colinear with the genomic sequence, as determined by DNA sequencing and restriction mapping, indicating that there are no intervening sequences. The lengths of the cDNAs are consistent with the estimated RNA size of 2.4 kb (see Fig. 3). The structure of the cloned breakpoints of the *usp*^{KA21} genomic DNA is shown beneath the wild-type restriction map. Thick lines, *XR2C* DNA; thin lines, non-*XR2C* DNA. Sequence analysis indicates that the *usp*^{KA21} breakpoint occurs in the region coding for the second finger of the putative DNA-binding domain of *XR2C* at nucleotide positions 661–663 within codon Cys 167 (see Fig. 2a). Restriction enzymes: R, *EcoRI*; S, *Sall*; X, *XhoI*; B, *BamHI*; P, *PstI*; H, *HindIII*. b, Southern blot of Canton S wild-type DNA digested with either *EcoRI* or *XhoI* was probed at high stringency with PB and reveals that *XR2C* is a single copy gene that lies on an 8.0-kb *EcoRI* and 4.5-kb *XhoI* fragment. c, Southern blot of genomic DNAs from various species was probed at low stringency with PB and reveals the existence of a few cross-hybridizing species in all organisms tested except for yeast. In the human lane, two main bands appear, of about 15 and 9 kb. These bands correspond to the bands seen when the DNA-binding domain of hXR2C is used as a probe⁶ DNA size markers (kb) to the left.

METHODS. Standard methodology was used for isolation of genomic DNA and Southern blotting¹⁸ and conditions for high and low stringency hybridization were as described elsewhere¹⁹. The Southern blot of genomic DNA from various species was purchased from Clontech (Palo Alto) and probed under low stringency conditions¹⁹. The *usp* library was made by digesting 10 µg genomic DNA from *usp*^{KA21}/FM7 females, size-fractionating the resultant fragments on an agarose gel, and isolating fragments of sizes 2–7 kb by electroelution. The DNA was packaged using LambdaZap II, *EcoRI*-digested phage arms (Stratagene). Phage were screened with a labelled 8-kb *EcoRI* fragment and plaque-purified candidate phage were converted to plasmids by the addition of helper phage R408 (Stratagene).

TABLE 1 Number of flies

Allele	<i>usp</i> y ⁺	<i>usp</i> y ⁺	<i>usp</i> +	<i>usp</i> +	Relative viability
	p[XR2C ⁺]	TM3	p[XR2C ⁺]	TM3	
<i>usp</i> ^{VE653}	154	0	128	116	1.2
<i>usp</i> ^{VE849}	126	0	124	90	1.0
<i>usp</i> ^{KA21}	100	0	140	87	0.7

XR2C genomic region specifically complements *usp* mutants. Complementation tests were performed by crossing XX *usp*/FM7 (ref. 14) females to +/Y; p[XR2C⁺]/TM3 males. Surviving *usp*/Y flies were scored with regard to their third chromosome genotype. Numbers indicate number of mutant flies that survived to adults. Columns are as marked. Rows indicate the particular *usp* allele tested. The alleles of other loci tested¹¹ were *l(1)2Cc*², *l(1)2Ce*¹, *l(1)2Ce*², and *l(1)2Ce*³. Alleles from the *l(1)2Cb* complementation group had previously been shown to be outside the genomic walk and were not tested (E. Fyrberg, personal communication). Relative viability was measured by comparing the number of rescued mutant males (*usp*/Y; p[XR2C⁺]/+) with the number of transposon-containing *usp*⁺ siblings (*usp*/+, p[XR2C⁺]/+). The relative viability of the mutant males with the transposon was near unity for each allele. The P-element germ-line transformation vector is pUChsneo (ref. 15) and contains the 8-kb *EcoRI* fragment inserted into the polylinker. G1 progeny of surviving, injected embryos were selected on *Drosophila* instant food containing 250 µg ml⁻¹ G418 as described previously¹⁶. One line shown to contain an insert by Southern analysis and resistance to G418 had an insert that was mapped by segregation to the third chromosome and balanced over TM3. Mutations in the 2C region had been generated previously¹⁷. Complementation was performed by crossing XX female flies of genotype *usp*/FM7; +/+ with males of genotype +/Y; p[XR2C⁺]/TM3 in vials on standard corn meal, molasses, agar media¹⁸. Parental flies were placed on new food every 3 days.

containing the breakpoint reveals the *XR2C* coding region has been disrupted within the putative DNA-binding domain (Figs 1a and 2a). The region flanking the *XR2C* transcription unit seems to be unaltered. These data show that *XR2C* is mutated on the *usp*^{KA21} chromosome and suggest that *XR2C* corresponds to the *usp* locus.

To test this hypothesis, the 8-kb *EcoRI* genomic fragment (see Fig. 1a) containing the *XR2C* gene was placed, by germ-line transformation, onto a wild-type third chromosome. Complementation tests were performed by crossing the wild-type copy of *XR2C* into mutant backgrounds and scoring for survival of the otherwise lethal class of flies. The 8-kb *EcoRI* fragment of *XR2C* specifically complements the three *usp* alleles (Table 1), but not four alleles tested from other complementation groups at position 2C (data not shown). The relative viability of the rescued flies is close to unity for all three alleles, indicating that the 8-kb genomic fragment containing the *XR2C* transcription unit is sufficient for *usp* function.

The *usp* locus has been previously characterized for its zygotic and maternal germ-line functions¹⁰. Removal of both maternal germ-line and zygotic *usp* expression results in an embryonic segmental abnormality involving a cuticular defect posterior to the eighth abdominal denticle belt. A zygotic wild-type copy of *usp* rescues the phenotypic consequences of a lack of maternal *usp* product and results in apparently normal development. Removal of zygotic expression from the progeny of *usp*/+ mothers results in a milder defect. These *usp*/Y embryos die as early second instar larvae with an additional set of posterior spiracles and filzkörper, thought to be due to the persistence of cuticular structures from earlier molts. Because of the epistasis of early death over other phenotypes, it has not been determined if this gene is needed for later functions. As a first step towards determining whether *usp* might be required at later stages, we examined the developmental profile of *usp* RNA expression (Fig. 3). Northern analysis reveals a single 2.4-kb transcript throughout the life cycle, with highest levels in adults and

FIG. 2 The nucleotide and predicted amino-acid (single-letter code) sequences of XR2C compared with the predicted amino-acid sequence of other members of the nuclear receptor superfamily. *a*, The complete nucleotide sequence of pXR2C8, the longest of five independent cDNA clones isolated by screening third instar larval imaginal disc and pupal cDNA libraries with the PB probe at high stringency. The predicted amino-acid sequence starts with methionine at nucleotide 163 and ends with a stop codon at nucleotide 1,689. The initiator methionine conforms to the *Drosophila* consensus sequence for translation initiation²⁰. An upstream, in-frame stop codon is underlined, implicating the first AUG codon as the start of initiation. Also underlined is the putative DNA-binding domain. The breakpoint of *usp*^{KA21} occurs within the putative DNA-binding domain at the codon for Cys 167 (arrow) and results in a deletion of nucleotide positions 661–663 (boxed nucleotides). Further, the chromosomal break juxtaposes a stop codon immediately after the codon for Thr 166. *b*, Alignment of the predicted amino-acid sequences of the *usp* and *hRXRα* protein products. Maximal alignment was determined using the local homology algorithm of Smith and Waterman on the BESTFIT program²¹. Symbols used for amino-acid comparisons are: (—), amino acid identity; (:), close evolutionary relationship; (.), distant relationship; no symbol, very distant relationship as defined by Dayhoff and normalized by Gribskov²². Note the high identity in the DNA-binding domain and in clusters of the putative ligand-binding domain. The *usp* amino-acid sequence contains two insertions of glycine-rich sequences before and in the middle of the putative ligand-binding domain (amino-acid positions 192–232 and 325–350). *c*, Comparisons of the predicted amino-acid sequence of the *usp* product with the predicted sequences of other nuclear receptors from both vertebrates and invertebrates. The region marked 'DNA' is compared with the 66–68-amino-acid DNA-binding domain and the region marked 'Ligand' is compared with the region following the DNA-binding domain which shares the greatest alignment with other nuclear receptors. The carboxy-terminal sequence of KNRL shares no significant sequence similarity with other receptors and, consequently, no alignment was attempted. Note the striking similarity in the DNA-binding and putative ligand-binding domains between the *usp* product and hRXR, much greater than that between other identified receptors.

METHODS. Screening, isolation and sequencing of candidate cDNAs were performed as described elsewhere²³. The sequence data were assembled and comparisons were performed using programs of Devereux *et al.*²¹. Numbers indicating amino acids are as detailed from USP (this report), hRXR α (ref. 6), hRAR α (ref. 4), E75A (ref. 24), KNRL (ref. 8), and hGR (ref. 25).

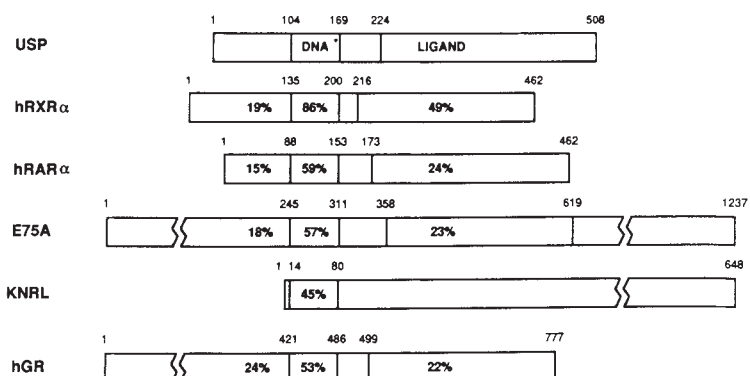
 b

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USP      1 MDCNQDQASFLRSLHKEEVKPDISQLNDSNSSFSPKAEVSVPFMQAMSMVHVLPGSNSASSNNN....SAGDAQMAQAPNSAGGSA 85
hRXR    19 LTPSTGRGSMAPASLHPSLGPGLSPGGL.HLSPITLSSPINGMGPFFSVISSMGPMSMVPPTTLGFGTSPQLSSPMMPVSSSEDI 107
USP      86 AVQ.....QQYPNHLPSGSKHLCSICGDRASGKHGYGVSYCEGCGFFKTRVRKOLTYACRENRCNIDKQRNRNCOYCRYQKCLT 166
hRXR    108 KPPLGLNGLVKYPAPHSNPMASFTKHCICGDRSSGKHGYGVSYCEGCGFFKTRVRKOLTYTCRDNKCOLIDKQRNRNCOYCRYQKCLA 197
USP     167 CGMKRAVEAQEERQGRNARGLSASGGGSSSPGVSGGSSSGGGGGGGVSGMGSGNSDDFMNTNSVRDPSIERIEAEQRAETQCGD 256
hRXR    198 MGMKRAVEAQEERQGRGKDRNEEVES.....TSSANEDMPVERILEAEAVEPKTET 248
USP     257 RALTFLRVGPYSTVPQDYGKVASLCOVYNKQLFMVYEARMHPHFAQVPLDDQVILLKAAWIELLLANVAWCSIVSLDDGGAGGGGGGL 346
hRXR    249 YVEANMGLNPSSNDP.....VTNICQAADKQLFTLEWAKRPIHFSLEPLDDQVILLRAGWELLTA..... 311
USP     347 GHGGSFERRSGPLGQQQLFNLQSFYHRRNSAIKAGYSATFDRLISELSVKMKRLNDRRESLCKLAIILYNPDIRIGKSRAEIEMKREKV 436
hRXR    312 ...SFSHRISAVK.DGILLATGLVHRRNSAHSAGVATFDRLVELVSKRMQRMOKTLEGLCRATLVLPNDSKGLNSPAEVEALREKV 396
USP     437 YACLDEHCRLEHPGGDGRFAQLLLRLPALRSISLCKQDHLFLFRITSDRPLEELEFLEQLEAPP 502
hRXR    397 YASLEAYCKHKYPEOPGRFAKLLRLPALRSIGLCKLEHFFFKLIGDPTIDTFLMELMELAPHOMT 462

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C



1 GGACACGGTGGCGTTGGCAAAGTGAACCCCAACAGAGAGGCGAAGCGAGCCAGACACACCATACACAGGAAGACAGCGACA 90

91 AGAAACCGGTAGGCGAGGAGGCGCTCCGCCCATCTCTCAATATACCCAGCACCACATCAAGCCGAGBAGACACCTGCACCA 180

181 GACGCGCAGCTTTCGGTGAGCCATCAAGGAGAGCTCAAGCCGCAGCTCTCCGAGTGAACGACGACAAACACGACAGCTTTCGCC 270

271 AAGCGCGAGAGTCCCGTCCCTCATGCGAGGCCATGCTAGGTCGACGTGCTGCGGCTCCCACTCCGCGAGTCTCAACAAACAGC 360

361 GCTGGAGATGCCAAATGGCGAGGCGCCCAATTCCGTGGAGGCTGCGCCGCTCGAGCTCCAGCAGCATTCGCCCTAACCATCG 450

451 CTGAGCGCGACGACCACTCTGCTTATTTCGGGGATCGGCGCAGTGGCGAAGCATCGCGGTGACAGCTGTGAGGGCTGCAAGGCG 540

541 TTCCTTAAGCAGCATGCGCAAGGATCTCACATACGCTTGACGGGAGAACCCGCACTGCATCATAGACAGCGGACAGCAAGCTGCG 630

631 CAGTACTGCCGTACCCAGAAGTGCCTAACCTGGCGGATCAAGCGCGCAAGCGGCGAGGCTCAACGCGCGCCGCAATCGCGCG 720

721 GGTAGGCTCAGCGCAGCGGAGGCGGAGTACGCGTTCAGGTCGATAGCGCGATCAGCTCTCAAGCGGAGGAGGAGGCGCGGCT 810

811 TCTGGCGGAATGGCGACGCGCAAGGTTCTGATGACTCTATGACCAATACGCTCTCAGGAGGATTTCCGATCGAGCATCATAGAGGCC 900

901 GAGCAGCGAGCGGAGACCCAATGCGGAGCTGCTGACGCTGACGCTTCTGCGGCTTGCTCCATTCCAGCTCCAGCGGACTACAAGGT 990

991 GCGGTGTCGCGCTGTGCCAAGTGTCAACAAAGCACTCTTCCAGTGTGCAATACGCGCGCATGATCGCGCACTTTCGCCAGTGTGCA 1080

1081 CTGAGCAGCAGGTGATTTCTGCGAAGCCGCTTGATGAGCTGCTCTATGCAACGTCGGCTGGTGACAGTATCTTTCGCTGGATGAC 1170

1171 GCGCGTGCAGCGCGCGCGCGGCTGAGCTAGGCGCAGATGCTCTTGTAGGACGATGACCGCGGCTTCAGCCCAAGCAGCTGTCTCT 1260

1261 AACCAAGAGCTTCTCGTACCATCGACACAGTGCAGTCAAGCGGTGTCAAGCCATCTTCGACGCGCATATGTTCGAGCTGAGTGTAAAG 1350

1351 ATGAAGCGGCTGAATCTCGACGACGCGGAGCTGTCTGCTTGAAGGCCATCATCTGTACAAACCGGACATACGCGGATCAAGAGCG 1440

1441 GCGGAGATCGAGATGTCGCGGAGAGGTTAGCTGTGCTTGGCAGGAGCAGTCCGCGCTGGAACATCCGCGCGACGATGAGCTTTCGCA 1530

1531 CAACTGCTGCTGCGTCTGCCCGCTTGTGCATGATCAGCTGAAGTGGCAGATCACCTGTCTCTTCGCGATACCCAGCAGCGCGCG 1620

1621 CTGAGGAGGCTTTCTCGAGCAGGCGCGCGCGCCGACCGCGCTGCGGATGAAGCTAGGCTAGGCTCCGAGCTCTAAAGTGCCTG 1710

1711 CCGGCTTCCATCCGAAAGTGTTCATGTGATGGGCTTTGTTCGATTTCTCTCTTCTATCTTCCTTATTCCTCAAAAGCCCCCTAAT 1800

1801 ATATCGCAAAATGCTGATGATATGTTTATTTTTCATTAACCAATATATTTATTTATGATATGAAAGATGTTTCTCTTAAGAT 1890

1891 GAAGATGAGCTCTCTGAGGCTTTATGTCGCGGATGAAGCAAAACAAATCAACTCAAACTCGAAGACCAAAACAGCAGCAAGAA 1980

1981 ATCCACACAGCAAGCAATGAAGTAAAGTCAAACTAAAGCTAAAGCATGAAGATATTAATAATCAAGTAAATATATGATCATGAT 2070

2071 ATCTCAGAGCTAGTGAAGATACAACTTACGATATAATATATGTACAGAGCGGCATCTCGGCTGGTGGCGGCTTCTTAACCAAT 2160

2161 TGTAATCATTTTAAACATAATATACCAAAACGTTATCAATAGATCGGAGTACAAATAACCCGACGAAACCAACAAATATATC 2250

2251 TATGATAAAAAATATAGCTGCATACGCAAAAAAAAATAAAAAATAAAAAATAAAAA 2311

uspK21 breakpoint

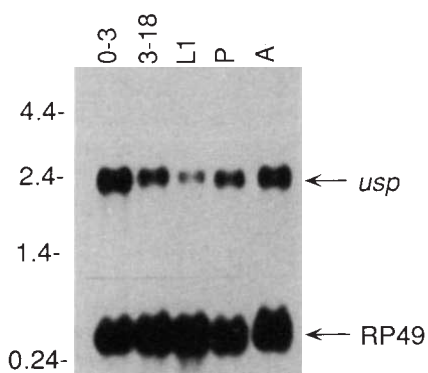


FIG. 3 Developmental expression of *usp*. A northern blot of poly(A)⁺ mRNA isolated from different stages of the *Drosophila* life cycle was probed with PB and revealed that *usp* gives rise to a single class of RNA of 2.4 kb throughout the *Drosophila* life cycle. The levels of the 2.4-kb RNA are roughly equivalent at all stages examined. Lanes 0–3 and 3–18 contain RNA extracted from embryos collected 0–3 and 3–18 h after egg laying, respectively. L1, RNA from first instar larvae; P, RNA from pupae; and A, RNA from adult males and females. As a quantitative control, the blot was stripped and reprobed with labelled DNA coding for the ribosomal protein RP49 which is expressed at roughly constant levels throughout development²⁶. RNA size markers (kb) to the left.

METHODS. The staged organisms were collected and *Drosophila* mRNA was isolated and poly(A)-selected by standard methods¹⁸. Poly(A)⁺RNA 10 µg were loaded per lane.

embryos and lower levels in larvae. Expression of *usp* after the lethal phase of *usp* mutants suggests a continuing role for *usp* through many developmental stages.

We demonstrate that an RXR-like molecule plays an important part in embryonic and post-embryonic development. The marked similarity between the products of the *usp* and *hRXR* genes indicates that the RXR subfamily is of ancient origin, existing before the divergence of insects and vertebrates. The inability to isolate a closely related RAR gene may reflect fortuitous restriction enzyme sites in the gene sequence or, more likely, an earlier origin of the RXR subfamily or greater subsequent rate of divergence of the RAR genes. We have demonstrated here that at least one member of the RXR subfamily is necessary for proper development of a metazoan organism. The homology between the DNA-binding domains of the *RXR* and *usp* gene products suggests that these proteins recognize similar DNA sequences. The similarity in the putative ligand-binding domain is roughly the same as that between the human glucocorticoid and mineralocorticoid receptors, which respond to an overlapping set of ligands¹². Metabolic studies indicate that *Drosophila* do not require vitamin A or its precursor β -carotene except for visual function and that they are incapable of synthesizing them *de novo*¹³. Moreover, preliminary studies using a cotransfection assay in Schneider cells, developed to characterize the vertebrate homologue RXR α ⁶, indicate that the product of the *usp* gene does not activate transcription in response to retinoic acid (data not shown). Together these data suggest that the critical endogenous ligand to which the *usp* gene product responds is not retinoic acid but may be a structurally similar molecule synthesized by an alternative metabolic pathway. □

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Studying single living cells and chromosomes by confocal Raman microspectroscopy

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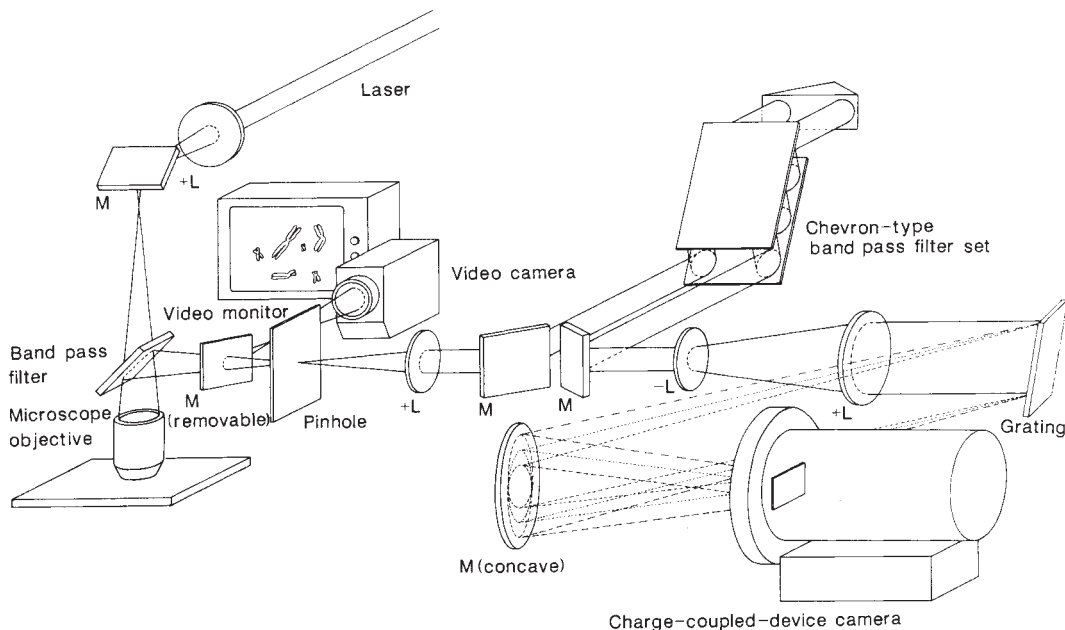
MANY indirect methods have been developed to study the constitution and conformation of macromolecules inside the living cell. Direct analysis by Raman spectroscopy is an ideal complement to techniques using directly labelled fluorescent probes or of indirect labelling with mono- and polyclonal antibodies. The high information content of Raman spectra can characterize biological macromolecules both in solution and in crystals^{1,2}. The positions, intensities and linewidths of the Raman lines (corresponding to vibrational energy levels) in spectra of DNA–protein complexes yield information about the composition, secondary structure and interactions of these molecules, including the chemical micro-environment of molecular subgroups. The main drawback of the method is the low Raman scattering cross-section of biological macromolecules, which until now has prohibited studies at the level of the single cell with the exception of (salmon) sperm heads, in which the DNA is condensed to an exceptionally high degree³. Ultraviolet-resonance Raman spectroscopy has been used to obtain single cell spectra (ref. 4; and F. Sureau and P. Y. Turpin, personal communication), but in this method absorption of laser light may impair the integrity of the sample. We have avoided this problem in developing a novel, highly sensitive confocal Raman microspectrometer for nonresonant Raman spectroscopy. Our instrument makes it possible to study single cells and chromosomes with a high spatial resolution ($\leq 1 \mu\text{m}^3$).

The configuration of our instrument (fig. 1) was carefully optimized to obtain a maximal signal throughput ($\sim 15\%$ of the scattered light collected by the microscope objective is actually detected). Light detection by the liquid-nitrogen-cooled charge-coupled-device camera is virtually photon-noise limited. Incident light of 660 nm is used to prevent degradation of the sample in the focused beam, a phenomenon observed with chromosomes and cells using the 514.5-nm line of an Argon-ion

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FIG. 1 Confocal Raman microspectrometer. Laser light of 660 nm from a DCM operated model 375 B Spectra Physics dye laser is focused on the object under investigation by a high numerical aperture microscope objective. Light scattered by the object is collected by the same objective, and coupled into the spectrometer through a pinhole, which enables confocal detection¹⁰. Lateral spatial resolution in the CRM is determined by the laser focus dimensions and is $\leq 0.5 \mu\text{m}$. A pinhole of $100 \mu\text{m}$ diameter leads to a depth resolution of $1.3 \mu\text{m}$. The spectrometer consists of a chevron-type dielectric band-pass filter set, which combines efficient stray light suppression (by a factor 10^9) with a high transmission (80–90% in the spectral interval $600\text{--}1,800 \text{ cm}^{-1}$) for Raman light (G. J. Puppels, A. Huizinga, H. W. Krabbe, H. A. de Boer, G. Gijsbers and F. F. M. de Mul, manuscript in preparation), and a wavelength dispersion stage. A liquid-nitrogen-cooled charge-coupled device camera (equipped with an English Electric Valve Co. P8603 B charge-coupled device chip, Wright Instruments Ltd) is used for signal detection. It combines a high quantum



efficiency ($\sim 40\%$ at 700 nm) with almost noise-free operation (negligible dark-current and a read-out noise of r.m.s. 10 electrons, equalling 10 detected photons). The spectral resolution of the microspectrometer is $\sim 6\text{--}7 \text{ cm}^{-1}$. Abbreviations: M, mirror; L, lens.

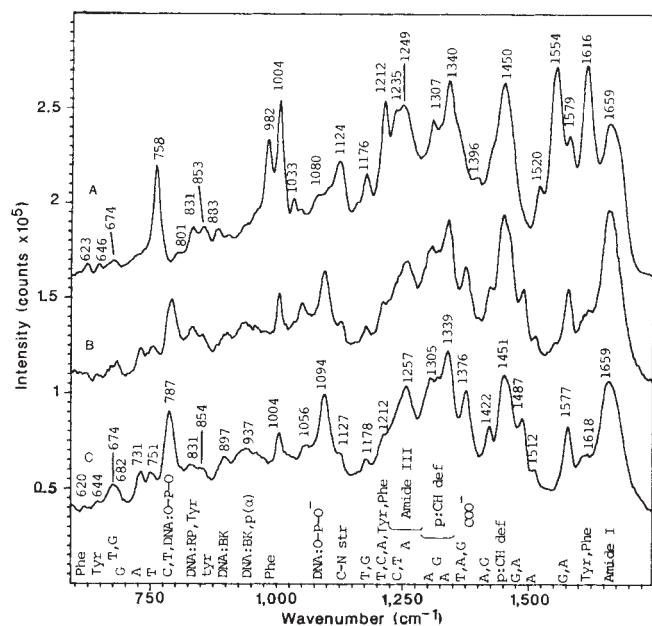


FIG. 2 Raman spectra of a single intact cell and a single metaphase chromosome. Spectrum A obtained from a cytoplasmic region of a human eosinophilic granulocyte. Spectrum B from the nucleus of the same cell, C obtained from a chromatid of a metacentric Chinese hamster lung cell (V79) metaphase chromosome. Intensity scale is for A (for B it is multiplied by 5, for C by 1.25). Spectra shifted along the ordinate for clarity. Raman line assignments are for nucleus and chromosome spectrum (B, C) and based on assignments in^{5,6,11–13}. Water (A, B, C) and substrate (C) signal subtracted. Abbreviations: T, thymine; C, cytosine; A, adenine; G, guanine; RP, ribophosphate; BK, backbone; p, protein; α , α helix; Tyr, tyrosine; Phe, phenylalanine; def, deformation; str, stretching. Experimental conditions: $\times 63$ Zeiss Plan Neofluar water-immersion objective (NA 1.2); laser power 5 mW (A, B), 10 mW (C); measuring times 150 s (A, B) or 900 s (C); granulocyte on poly-L-lysine-coated fused silica (fs) substrate in standard PBS isolation method of ref. 14; chromosome on fs substrate in hypotonic buffer (4 mM MgCl_2 , 10 mM Tris and 10 mM NaCl), isolation method of ref. 15.

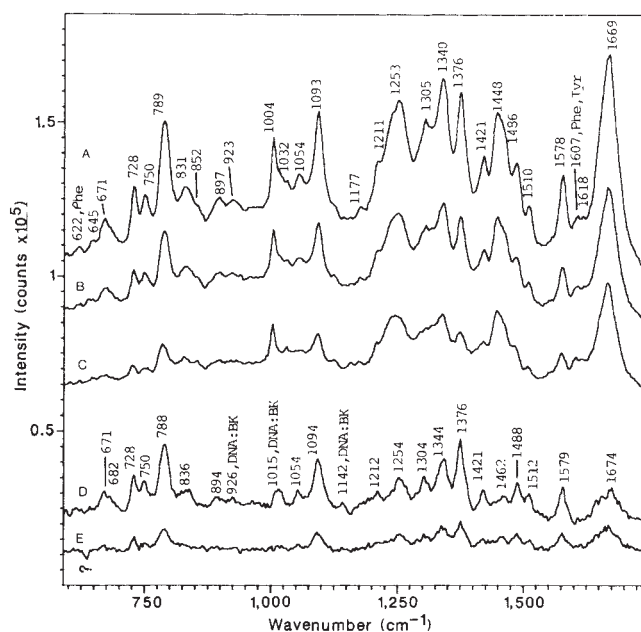


FIG. 3 Raman spectra obtained from polytene chromosome II of *Chironomus thummi thummi* (salivary gland), shown in Fig. 4. Spectrum A, band (arrow 1); B, interband (arrow 2); C, telomere (arrow 3); D, difference spectrum A – C scaled on 1450 cm^{-1} protein line; E, difference spectrum A – B (as in D, multiplied by 2). Spectra shifted along ordinate for clarity. Raman line assignments as in Fig. 2 spectra B and C. The line marked '?' is most probably artefactual and caused by detection of a cosmic ray event in the recording of spectrum B. Buffer signal subtracted (A, B, C). Experimental conditions: $\times 63$ Zeiss Plan Neofluar water-immersion objective (NA 1.2/0.12–0.22); laser power, 15 mW; measuring time, 600 s; chromosome on fs substrate in PBS with added divalent cations ($1 \text{ mg ml}^{-1} \text{ MgCl}_2$ and $1 \text{ mg ml}^{-1} \text{ CaCl}_2$) covered by fs coverslip. Fixation procedure according to ref. 8.

laser (G. J. Puppels, J. H. F. Olminkhof, G. M. J. Segers-Nolten, C. Otto, F. F. M. de Mul and J. Greve, manuscript in preparation).

The data reported here exemplify the spatial resolution and sensitivity of the microspectrometer. The spectra shown are not smoothed and were recorded with the samples at room temperature. Figure 2 shows spectra obtained from the cytoplasm (a) and the nucleus (b) of an intact human eosinophilic granulocyte. The nucleus spectrum consists of lines that can be assigned to DNA and protein vibrations and strongly resembles reported spectra of isolated chromatin^{5,6} and also spectrum 2C, which was obtained from a single metaphase chromosome. The cytoplasm spectrum differs dramatically from that of the nucleus. This is most probably due to signal contributions from the many cytoplasmic granules, the Raman lines of which have so far to be assigned. The amount of DNA present in the measuring volume during the recording of spectrum 2C was estimated to be ~50 fg, corresponding to 50×10^6 base pairs. The high quality of the spectrum indicates that even much smaller amounts of material would suffice for recording good spectra.

The experiments with metaphase chromosomes are aimed at obtaining a better understanding of the origins of the banding patterns that appear after physico-chemical treatment and staining as first described by Caspersson *et al.*⁷ Figure 3 displays spectra obtained from a band (a), an interband (b) and a telomere (c) of a fixed polytene chromosome (figure 4). An interesting feature is the difference in the DNA/protein ratio between bands, interbands and telomeres. Figure 3d, e shows difference spectra scaled on the $1,450\text{ cm}^{-1}$ protein line. From the fact that only DNA lines remain, it is clear that this ratio is lowest in the telomere and highest in the bands. Whereas spectra obtained from different chromosome bands are very similar both in absolute signal intensity and relative intensity of the Raman lines, spectra from different interbands show marked variations, indicative of different DNA-protein ratios. In all cases this ratio is lower in interbands than in bands. We are presently investigating the possibility that these differences are related to local variations in transcriptional activity, by studying unfixed chromosomes as well. The microspectrometer is also being employed to study the presence and induction of left-handed Z-DNA in such chromosomes^{8,9}.

The use of 660-nm laser light enables Raman measurements on cells and chromosomes which have been labelled with fluorescein-conjugated antibodies, thus facilitating the prior localization of specific structures such as sites of transcription or replication by conventional fluorescence microscopy. The combination with (immuno)fluorescence techniques thus further increases the potentials of the CRM. Moreover the Raman

spectra could yield valuable information about the way samples (and experimental results) may be affected by the application of fluorescence techniques.

Finally, we expect a vast range of applications of this instrument in the analysis of small biological structures and possibly for monitoring biological events, such as the cytotoxic process mediated by natural killer cells, on a single cell level. □

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Histidine oxidation in the oxygen-evolving photosystem-II enzyme

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THE evolution of oxygen as a result of light-driven water oxidation occurs in plants and is catalysed by photosystem-II (PS-II). A manganese-cluster probably acts both as the active site and as a charge-accumulating device (for a review, see ref. 1). The enzyme cycle involves five redox states which are denoted as S_0 - S_4 , depending on the number of positive equivalents stored². Oxygen is released after formation of the transient S_4 state. Ca^{2+} is an obligatory cofactor in this process and its depletion inhibits the enzyme cycle at the step before water oxidation, that is, after formation of the S_3 state³. In chelator-treated, Ca^{2+} -depleted PS-II, a new electron paramagnetic resonance (EPR) signal arising from a formal S_3 state has been reported⁴. It was suggested that the S_3 EPR signal could originate from the oxidation of an amino acid, interacting magnetically with the manganese cluster⁴. There are only a few examples of amino-acid oxidation in enzyme chemistry and these are limited to tyrosine⁵ and tryptophan⁶. Here we report evidence that the S_2 to S_3 transition occurring in Ca^{2+} -depleted PS-II corresponds to the oxidation of histidine.

As reported previously⁴, we observed two new EPR signals in Ca^{2+} -depleted PS-II. First, a signal at $g = 1.98$ consisting of many lines (>26) spread over 1,700 gauss which is stable in the dark at 0 °C and is attributed to a modified S_2 state. This multiline signal is a modified form of that arising from the S_2 state in the active enzyme⁷ and it is likewise ascribed to a mixed-valence manganese cluster having a spin- $\frac{1}{2}$ ground state⁴. The second new EPR signal, which is attributed to a formal S_3 state, is centred at $g = 2.004$, with a linewidth of 164 G. Previously we

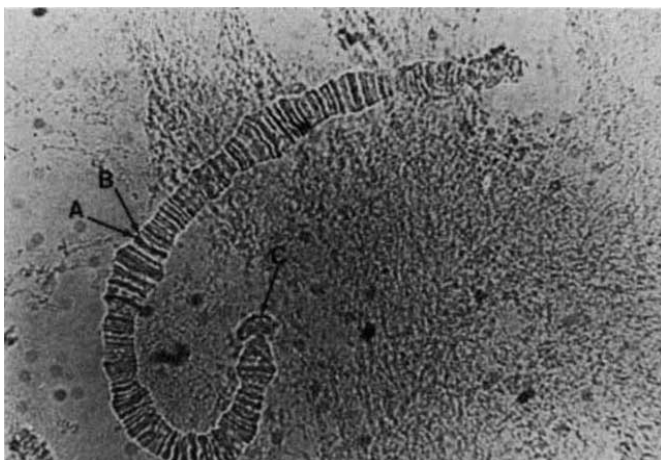


FIG. 4 Polytene chromosome II of *Chironomus thummi thummi* (salivary gland). Indicated are the positions where the spectra of Fig. 3 were obtained. Bar, 10 μm .

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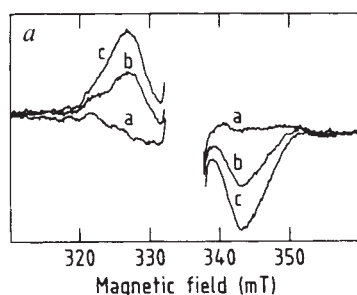
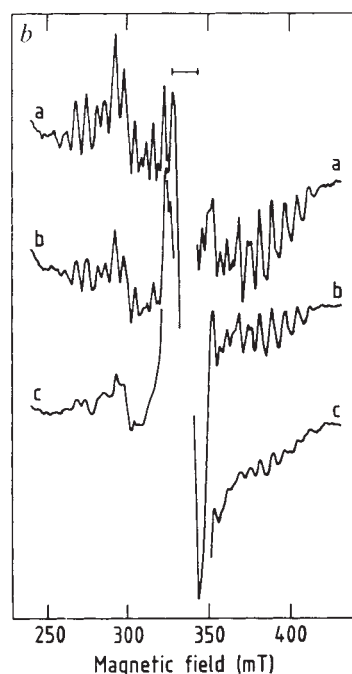


FIG. 1 EPR spectra from Ca^{2+} -depleted, EGTA-treated PS-II membranes and the effect of flash illumination. *a*, Spectrum *a* corresponds to a dark-adapted sample. Spectrum *b* was recorded after one flash and spectrum *c* after 4 flashes at room temperature. The centre parts of the spectra have not been drawn because of the overlapping signal arising from the oxidized tyrosine 161 of the D_2 protein. Instrument settings: microwave frequency, 9.44 GHz; temperature, 15 K; modulation amplitude, 3 G; microwave power, 10 mW. Phenyl-*p*-benzoquinone (1 mM), dissolved in dimethylsulphoxide, was used as an artificial electron acceptor. *b*, Spectra *a*, *b* and *c* correspond to the same samples as in panel *a* with instrument settings allowing resolution of the multiline signal, that is, modulation amplitude, 20 G; temperature, 10 K; microwave power, 20 mW. Bar represents 164 gauss and marks the position of the low-field peak and the high-field trough of the flash-induced signal after four flashes than after the first flash is mainly due to a rapid disappearance (half life ~ 300 ms as seen by ultraviolet experiments) of the signal in $\sim 40\%$ of PS-II centres during the time between flash and freezing of the sample. This disappearance is due in part to charge recombination with the electron stored on the acceptor side. Therefore four flashes are required to trap almost 100% of the light-induced state.

METHODS. PS-II membranes were depleted of Ca^{2+} by a treatment with NaCl and EGTA (ref. 4). The procedure used maintains the polypeptide



constitution of PS-II. EPR spectra were recorded with a Bruker ESP 300 X-band spectrometer equipped with an Oxford Instrument cryostat. Flash illumination was as in ref. 3. The time required to freeze the EPR sample after flash excitation was about 2 s. Concentration of the sample was ~ 2.5 mg chlorophyll per ml in 0.3 M sucrose, 10 mM NaCl, 25 mM MES pH 6.5.

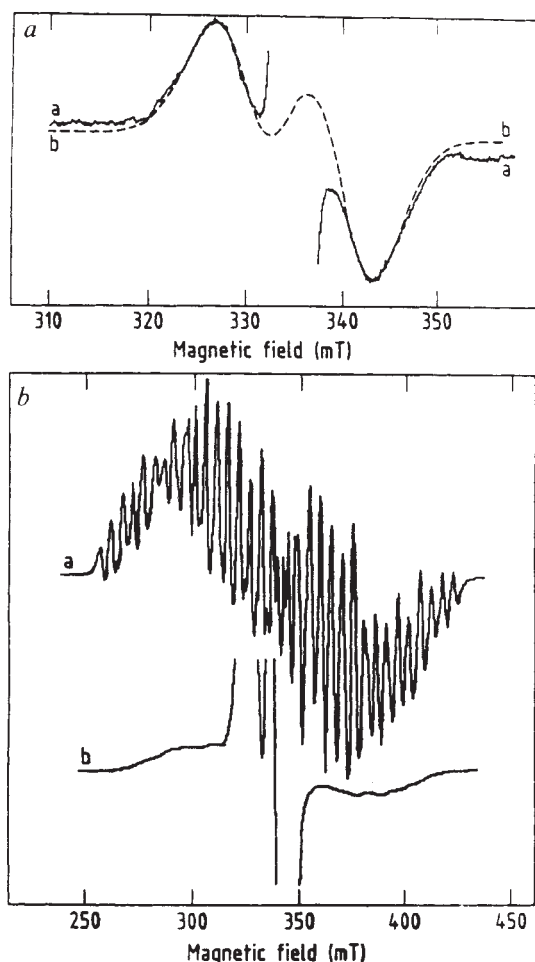


FIG. 2 Simulation of the S_3 EPR spectrum. *a*, Spectrum *a* shows the experimental S_3 spectrum induced by flash illumination. Spectrum *b* shows the calculated spectrum for a radical ($S_{\text{rad}} = \frac{1}{2}$) interacting with a Mn system ($S_{\text{c}} = \frac{5}{2}$) that mimics the properties of the Mn cluster in the S_2 state. As the electronic structure of the Mn cluster responsible for the S_2 multiline EPR signal is not known, a simple phenomenological model involving a Mn tetramer ($S_{\text{c}} = \frac{1}{2}$, $I_1 = I_2 = I_3 = I_4 = \frac{5}{2}$, $A_1 = 140.1 \times 10^{-4} \text{ cm}^{-1}$, $A_2 = 70.0 \times 10^{-4} \text{ cm}^{-1}$, $A_3 = 49.3 \times 10^{-4} \text{ cm}^{-1}$, $A_4 = 45.4 \times 10^{-4} \text{ cm}^{-1}$ where I_i and A_i are respectively the nuclear spin and isotropic coupling constant of atom i) was used here as the $S_{\text{c}} = \frac{5}{2}$ system. This model gives a multiline signal (spectrum *a*, panel *b*) with an average hyperfine spacing of 55 G, which is similar to that observed for the S_2 multiline signal in the inhibited enzyme. The parameters for the model Mn tetramer are taken from the work of J. Bonvoisin, J.-J. Girerd and J.-L. Zimmermann (manuscript in preparation). The type of magnetic interaction occurring between the free radical and the Mn-cluster may be dipole-dipole and/or electron exchange. It seems unlikely however, that the interaction can be explained in terms of a simple dipolar coupling, as the relaxation properties of the Mn multiline signal, which are rapid relative to those of an organic free radical, would result in only a broadening, rather than a splitting, of the radical signal (for example, refs 8, 22). Thus it is likely that the interaction is dominated by a weak electron exchange. The hamiltonian $\mathcal{H} = g_{\text{rad}} \beta S_{\text{rad}} \cdot H + g_{\text{c}} \beta S_{\text{c}} \cdot H + (I_1 \cdot A_1 + I_2 \cdot A_2 + I_3 \cdot A_3 + I_4 \cdot A_4) \cdot S_{\text{c}} + J \cdot S_{\text{rad}} \cdot S_{\text{c}}$, with isotropic g , A , and J (J is the exchange coupling constant and the other parameters have their usual meanings) tensors was diagonalized and the energy levels and EPR transition probabilities were calculated as in refs 23 and 24. The following simulation parameters were used: $g_{\text{rad}} = 2.012$, $g_{\text{c}} = 1.965$, $J = 0.0063 \text{ cm}^{-1}$. Other examples of exchange interactions of this magnitude between organic free radicals and metals have been reported^{23,25}. The resulting stick spectrum was convolved with gaussian lineshape functions with $\Delta H_{\text{rad}} = 43 \text{ G}$, $\Delta H_{\text{c}} = 40 \text{ G}$ to yield spectrum *b*. These large linewidths for the two interacting spins had to be included to reproduce the shape of the experimental spectrum. Such broadening could arise from some dipolar coupling. A distribution of J values over a limited range could also result in such broadened lines²⁶. We note that the g value of oxidized histidine²⁷ ($g = 2.009$) is similar to that of the free radical involved in the proposed magnetic interaction. *b*, Spectrum *a* is the calculated EPR spectrum of the hypothetical Mn tetramer which is used as the $S_{\text{c}} = \frac{5}{2}$ system in the simulation of the S_3 signal. Spectrum *b* is the same simulation of the S_3 signal as shown in panel *a* except that it is plotted on a field and amplitude scale allowing a comparison with the Mn multiline signal. The amplitudes of spectra *a* and *b* are scaled relative to the amplitudes of their biological counterparts (compare with Fig. 1*b* for example).

showed that this signal could be induced by continuous illumination at 0 °C and was accompanied by the disappearance of the modified multiline signal⁴. This reaction occurs with a high yield using flash illumination (60% after the first flash and ~100% after four flashes; Fig. 1). The high yield of the signal induced

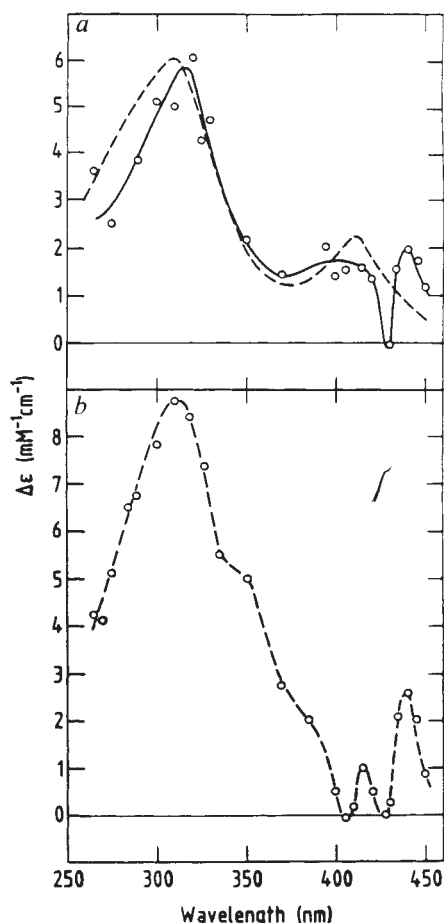


FIG. 3 Absorption changes induced by flash illumination. Absorption changes were measured with the apparatus developed by Joliot *et al.*^{28,29}. *a*, Symbols and solid curve: absorption spectrum due to the S_2 to S_3 transition in NaCl-EGTA-treated, polypeptide-reconstituted PS-II membranes. The spectrum was obtained as follows: first, the S_2Q_A to $S_3Q_A^-$ transition was measured in the presence of 10^{-5} M DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) in the 100–250 ms range after four flashes spaced 50 ms apart. Second, the Q_A to Q_A^- spectrum was measured in the presence of DCMU and 2 mM hydroxylamine (an artificial electron donor). Both spectra were normalized to the trough of the C-550 shift caused by Q_A reduction, that is, at 550 nm where no contribution of the donor side occurs¹⁰. Then the Q_A to Q_A^- spectrum was subtracted from the S_2Q_A to $S_3Q_A^-$ spectrum, resulting in a pure S_2 to S_3 spectrum. Hydroxylamine addition caused about ~20% increase in the Q_A reduction thus measured. This indicates that a fraction of centres could not achieve a stable charge separation. The reproducibility range for the S_2 to S_3 spectrum obtained by this procedure was better than one unit of the ordinate scale. Dashed line spectrum of the oxidized histidine radical at pH 9.2 redrawn from ref. 12. According to these authors, this spectrum corresponded to an $OH^\bullet$ adduct. If the same species occurred *in vivo*, it would represent an intermediate relevant to the water oxidation process. *b*, Spectrum of the S_1 to S_2 transition in untreated PS-II. The same procedure as in *a* was used, except that the DCMU experiment was run by averaging the change recorded in the 100 ms range after each flash in series of 9 (spaced 15 s apart), discarding the data from the first flash. This procedure was used to eliminate any contribution from centres in the S_0 state. The ordinate scale in both panels was calibrated by taking the value of $14 \text{ mM}^{-1} \text{ cm}^{-1}$ for the peak of the Q_A^- minus Q_A spectrum at 325 nm (ref. 30). The absorption changes were not corrected for the flattening effect. Therefore, the $\Delta\epsilon$ scale is strictly quantitative only around this wavelength. We note that the Q_A^- minus Q_A spectra obtained with treated or untreated PS-II were indistinguishable in the UV region (not shown).

by flash illumination suggests that it is unlikely to be due to a nonspecific photooxidation reaction.

In Fig. 1, the EPR signal induced by flash illumination is better resolved than that reported earlier⁴. It is now evident that the signal is split into two lines, rather than being simply a broad gaussian⁴. These spectral features and the microwave power saturation characteristics⁴, indicate that the signal arises from an organic free radical that interacts magnetically with a faster relaxing paramagnet (see for example, ref. 8 and Fig. 2 legend). The loss of the Mn multiline signal, which accompanies the formation of the S_3 signal, indicates that the Mn cluster is the faster relaxing paramagnet that interacts with the organic free radical.

The flash-induced signal can be simulated assuming a weak exchange interaction ($J = 0.0063 \text{ cm}^{-1}$) between an organic free radical and a hypothetical mixed-valence Mn tetramer having spectral properties analogous to the S_2 state (spectrum *a*, Fig. 2*b*). The simulation is of particular interest as it reproduces not only the asymmetric line shape of the S_3 signal (Fig. 2*a*) but also the collapse of the hyperfine pattern of the Mn signal (spectrum *b*, Fig. 2*b*). The simulation provides a simple, specific, theoretical model that can explain the EPR data. It shows that the straightforward interpretation of the S_3 signal, as an organic free radical interacting magnetically with the Mn cluster, is physically reasonable.

In our original report on the S_3 signal we did not rule out the possibility that it might arise from Mn oxidation. But this was considered as a rather remote possibility because it was difficult to accept that the new S_3 signal, which is essentially a spin- $\frac{1}{2}$ state, could be formed by a one-electron oxidation of a Mn cluster that already exhibits the spin- $\frac{1}{2}$ state multiline signal; also, it seemed unlikely that such a narrow, featureless signal could arise from a Mn cluster⁴. The new observation that the signal is split around $g = 2$, as well as being indicative of an organic radical, is even less reconcilable with it arising from Mn.

Figure 3*a* (solid curve) shows the near ultraviolet absorption spectrum for the S_2 to S_3 transition in the inhibited enzyme. As found in the EPR experiments, a single saturating flash caused absorption changes that amounted to ~60% of those obtained after four flashes. For comparison, the spectrum of the S_1 to S_2 transition, obtained using a similar procedure with untreated PS-II, is shown in Fig. 3*b* (note that this spectrum is very similar to that of the S_1 to S_2 transition reported previously^{9,10}). The spectrum of the S_2 to S_3 transition in the active enzyme of living algae was previously reported to have roughly the same peak position as that of the S_1 to S_2 transition, with an extinction coefficient smaller by about 25%, and a broader bandwidth¹⁰. The first of these features are exhibited by the S_2 to S_3 transition in the inhibited enzyme (Fig. 3*a*) but the broader bandwidth is not observed. It is possible that the same electron carrier is photo-oxidized in the S_2 to S_3 transition in both the inhibited and native enzyme. The minor spectral differences could be due to structural modifications in the inhibited enzyme and/or to the deconvolution procedures used in earlier work, which were necessary to obtain the S_2 to S_3 spectrum after two photochemical turnovers¹⁰. An electrochromic bandshift of chlorophyll *a* around 433 nm is also observed in the S_2 to S_3 spectrum (Fig. 3*a*), which is similar to that occurring on the S_1 to S_2 change (Fig. 3*b*), but which was absent in the S_2 to S_3 spectrum from the active enzyme¹⁰. This suggests that a net charge increase accompanies the S_2 to S_3 oxidation in the modified system and that no, or only partial, deprotonation occurs.

There are several different organic cofactors associated with the enzyme, namely chlorophyll, pheophytin, carotenoid and hydroquinone, any of which could give rise to the free radical. All of these can be ruled out because their well-known spectra are so different from the experimental spectrum. In the absence of any other candidates, amino acids seem to be the only likely alternative. Amino-acid oxidation is not unexpected as tyrosine

oxidation has already been demonstrated in this enzyme^{5,11} and because the enzyme must generate uniquely oxidizing conditions to oxidize its natural substrate, water. Indeed, the experimental spectrum of the radical (Fig. 3a, solid curve) is remarkably similar to that of oxidized histidine reported previously¹² (Fig. 3a, dashed line; see also ref. 13). No other oxidized amino acid¹⁴ has an ultraviolet spectrum similar to the experimental spectrum in Fig. 3a. We conclude that histidine probably undergoes oxidation in the S₂ to S₃ transition of the inhibited enzyme.

From the model of the PS-II reaction centre proposed by Trebst¹⁵ and the location of the Mn cluster discussed by Babcock *et al.*¹¹, the most likely candidates as the oxidized histidine are probably those on the lumenal side of the D₁ protein (that is, His 92, His 190, His 332, His 337). Electron spin echo envelope modulation experiments have recently shown nitrogen coupling to the Mn (V. J. DeRose, V. K. Yachandra, A. E. McDermott, R. D. Britt, K. Sauer and M. P. Klein, personal communication). This could reflect histidine binding as a ligand to the Mn as suggested earlier (for example, ref. 16). It is possible that the histidine shown to be oxidized here is a ligand of the Mn and that the proposed exchange coupling reflects a through-bond interaction. But the weakness of the exchange interaction may argue against direct liganding of the histidine to the Mn.

In most recent models it is suggested that Mn is oxidized on each of the four charge-accumulation steps^{1,11}. In fact, there is little evidence to support this idea¹. By contrast, experimental data from NMR¹⁷ and EPR¹⁸ relaxation studies and X-ray absorption spectroscopy¹⁹ were interpreted as indicating that Mn oxidation does not occur on the S₂ to S₃ transition in the functional enzyme. Indeed, the oxidation of an organic free radical (for example, histidine¹⁹) and its magnetic interaction with the Mn cluster had already been proposed to explain the disappearance of the S₂ multiline signal^{18,19}. In addition, it was pointed out earlier²⁰ that the midpoint potential of histidine (near 1.0 V; ref. 21) is consistent with its involvement as an oxidized intermediate in the functional water-oxidizing enzyme. An S₃ EPR signal could have remained undetected (because of, for example, increased broadening) in the functional enzyme, because of differences in the nature and magnitude of the magnetic interaction compared with those in the inhibited enzyme. Many of the properties of the S₃ signal in the inhibited enzyme reported here and earlier⁴ are consistent with those expected for the normal S₃ state. It is thus reasonable to propose that a histidine is oxidized on the S₂ to S₃ transition in the functional enzyme. □

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Three-dimensional structure of ribonuclease H from *E. coli*

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THE three-dimensional structure of RNase H from *Escherichia coli* was determined at 1.8 Å resolution by X-ray crystallography. The enzyme was found to belong to the α+β class of structures, consisting of two distinct domains. The structure implies a possible region interacting with a DNA–RNA hybrid. The Mg²⁺-binding site essential for activity is located near a cluster of four acidic amino acids—one glutamic and three aspartic acid residues. These residues are completely conserved in the homology alignment of sequences of RNase H and reverse transcriptases from retroviruses and retrovirus-like entities^{1,2}. The structural motif of β strands around the Mg²⁺-binding site has similarities to that in DNase I^{3–6}.

RNase H, which is found in various organisms from prokaryotes to eukaryotes, endonucleolytically degrades RNA moieties of a DNA–RNA hybrid in the presence of Mg²⁺ and produces 5'-phosphates at the hydrolysis sites⁷. Some findings^{8–9} suggest that it participates in DNA replication. As deduced from the DNA sequence of the structural gene, the enzyme consists of a single polypeptide chain of 155 amino acids¹⁰. Furthermore, Johnson *et al.*^{1–2} proposed from computer analysis of sequences that reverse transcriptases from retroviruses, including human immunodeficiency virus, may contain a segment homologous with RNase H from *E. coli* downstream of the RNA-directed polymerase domain. Viral RNase H has been suggested to be responsible for degradation of genomic viral RNA in the DNA–RNA hybrid so that minus-strand DNA is available as a template for synthesis of plus-strand DNA¹¹. Here we describe the three-dimensional structure of RNase H determined from X-ray diffraction of single crystals, and its functional implication.

Crystals of RNase H were obtained as reported previously¹². They were grown in the absence of Mg²⁺ which is essential for the enzymatic activity. The crystals belong to the space group P2₁2₁2₁ with unit cell parameters $a = 44.1$ Å, $b = 87.0$ Å, $c = 35.5$ Å and contain one molecule per asymmetric unit. They diffract X-rays beyond 1.8 Å resolution. Conventional multiple isomorphous replacement (MIR) was used for phase determination. The final statistics of structural determination are summarized in Table 1.

The main-chain folding of RNase H is represented in Fig. 1. The molecule has an irregular ellipsoidal shape with overall dimensions 50 Å × 45 Å × 40 Å. On constructing a distance map¹³ it was found to be divided into two domains. The principal domain is constituted from four α helices (αI, αII, αIV, αV) and one large β sheet consisting of three antiparallel β strands (βA, βB, βC) at the N terminus, as well as two parallel β strands (βD, βE). The β sheet shows the usual twist commonly found in many other proteins. The minor domain consists of one α helix (αIII) with roughly two turns and a following loop composed of about 10 residues. The joint between αII and αIII, whose axes make a kink at an angle of 35°, is composed of only

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TABLE 1 Structure determination statistics

Data set	Resolution (Å)	No. of independent reflections	No. of crystals	R_{merge} (%)	Average isomorphous difference (%)	Phasing power	R_{cullis}
CAD4 (for MIR)							
Native	2.5	5,000	1	‡	—	—	—
EMTS	2.5	4,586†	4	5.00	12.3	2.72	0.47
HgCl ₂ *	2.6	4,325	2	6.13	11.3	3.59	0.41
5-HgCl-uridine (1) *	2.5	4,825	3	6.21	20.5	3.12	0.44
5-HgCl-uridine (2) *	2.5	4,759†	4	5.36	17.3	2.58	0.55
BaCl ₂	3.5	1,889	1	‡	10.9	0.88	0.81
FAST (for refinements)							
Native	1.8	10,018	2	6.33	—	—	—
Resolution (Å)	14.81	8.70	6.15	4.76	3.88	3.28	2.50
No. of reflections	33	112	241	411	630	890	1,491
Figure of merit	0.97	0.96	0.92	0.88	0.81	0.75	0.63
							0.60
							0.71

Intensity data to 2.5 Å resolution were collected using a four-circle diffractometer (Enraf-Nonius, CAD4) on a generator with a sealed fine focus Cu tube. For refinement, another intensity data set at 1.8 Å resolution was collected with a FAST area-detector diffractometer on a GX21 rotating anode X-ray generator (Enraf-Nonius). The heavy atom parameters were refined using the program PROTEIN²⁴. $R_{\text{merge}} = \sum \sum |I_{h,j} - \langle I_h \rangle| / \sum \langle I_h \rangle$; $R_{\text{cullis}} = \sum ||F_{\text{PH}} \pm F_{\text{P}}| - F_{\text{H(calc)}}| / \sum |F_{\text{PH}} - F_{\text{P}}|$ (for centric reflections). Phasing power is the ratio of the root mean square (r.m.s.) heavy atom scattering factor amplitude to the r.m.s. lack of closure error. The initial model for RNase H was constructed by use of the interactive computer program FRODO²⁵ on an Evans and Sutherland PS390 graphics system. The model was refined with the program PROLSQ²⁶ running on a FACOM VP400E super computer. Occupancies were held fixed at 1.0 and water molecules which refined to B value $> 50 \text{ Å}^2$ were deleted from the model. The current R factor is 18% on all data from 5.0 to 1.8 Å and 20% on all data from 10 to 1.8 Å after restrained B factor refinement where 84 water molecules are included. The r.m.s. deviations from ideal bond lengths and 1–3 bond angle distances were 0.013 Å and 0.039 Å, respectively.

* Derivatives prepared in the presence of 2-mercaptoethanol. The major site of EMTS, HgCl₂ and 5-HgCl-uridine (2) lies near Cys 63. Another 5-HgCl-uridine (1) derivative, which was prepared at a different ratio of 2-mercaptoethanol to 5-HgCl-uridine, has a minor site in addition to the major site.

† Additionally include Bijvoet pairs.

‡ Entire data set collected from a single crystal.

a few residues, not including proline. Data base analysis shows¹⁴ no similar kink in the three-dimensional structures of other proteins. Consequently, the enzyme can be classified into an $\alpha + \beta$ structure¹⁵. The five-stranded β sheet and a set of two α helices (α II, α III) form a large cleft with the bottom of α I and α IV in the middle of the molecule. All tryptophan residues at 81, 85, 90, 104, 118 and 120 are concentrated around the cleft.

RNase H requires Mg^{2+} for activity, and the position of Mg^{2+} is assumed to be close to the catalytic site. To identify the

Mg^{2+} -binding site, a difference Fourier map ($\{|F_{\text{Mg}}| - |F_{\text{rec}}|\} \exp\{i\alpha_{\text{MIR}}\}$) based on MIR phases was calculated between the Mg^{2+} -bound crystal, which was grown in a solution containing Mg^{2+} , and the Mg^{2+} -free crystal. The map indicated a significant peak near the amino-end of α I and the loop connecting β A with β B (Fig. 1b). To obtain further evidence, we soaked crystals in a solution of metal ions such as Ba^{2+} , Co^{2+} and Ca^{2+} , whose atomic numbers are greater than that of Mg^{2+} . HPLC analysis of digestion products revealed that Ba^{2+}

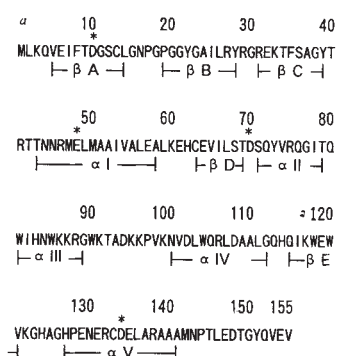


FIG. 1 a, Amino-acid sequence and secondary structure of RNase H from *E. coli*. The ranges of five helices and five β strands are shown underneath the sequence. *, the four acidic amino-acid residues, which are conserved completely among sequences in the homology alignment (see Fig. 15 in ref. 2) for retroviral reverse transcriptases, other reverse transcriptase-bearing entities present in the genomes of eukaryotes as well as for RNase H from *E. coli*. The amino acids forming the minor domain are roughly residues 80–100. b, Ribbon representation of the main chain folding of RNase H. The minor domain, consisting of α III and the loop leading to α IV, is located at middle right. All tryptophans, which are indicated by green bonds with residue numbers, are concentrated in a cleft surrounded by α II, α III, α IV and β E. The Mg^{2+} -binding site essential for the catalytic activity is shown near the bottom left. A yellow sphere indicates the position of Mg^{2+} bound to the site. The three aspartic acid residues and one glutamic acid residue are represented by orange spheres and yellow bonds with residue numbers.

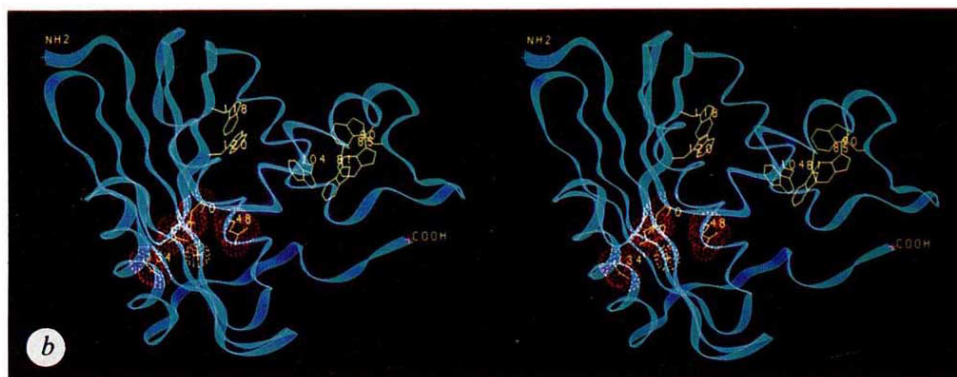
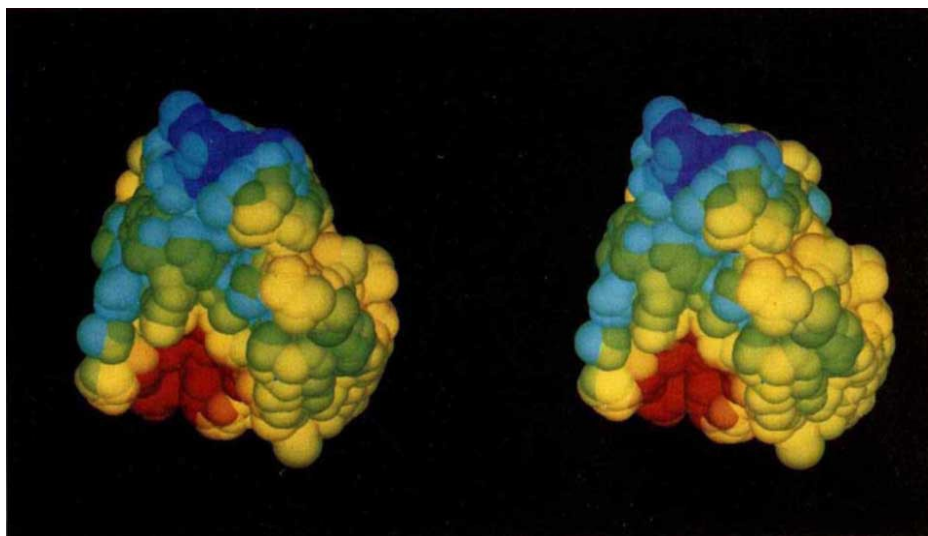


FIG. 2 Distribution of the electrostatic potential¹⁷ on the solvent-accessible surface of RNase H from *E. coli*. Colour codes for electrostatic potential values: red, below -0.1 V and blue, above $+0.1$ V. Other colour codes correspond to values between 0.1 and -0.1 V sequentially following the spectrum from blue to red. The catalytic activity of RNase H is optimum at pH 8.0, and hence the electrostatic potential was calculated in a condition where histidine residues are not protonated. The red region at the bottom indicates the Mg^{2+} -binding site, and the blue region indicates the basic minor domain. Both regions are supposed to interact with a DNA-RNA hybrid. This interface between the enzyme and the nucleic acid has been supported by recent NMR measurements (Y. Oda and H. Nakamura, unpublished results), in which all signals of the main chain affected by adding a DNA-RNA hybrid oligomer in a RNase H solution were assigned. All residues accompanying large changes in chemical shifts are



localized in a region including the Mg^{2+} -binding site, the basic minor domain and residues lying between them.

and Ca^{2+} are less efficient at promoting the digestion of RNA than Mg^{2+} (S. Iwai, M.I., K.M. and E. Ohtsuka, manuscript in preparation). The difference Fourier maps indicated a single, common, significant peak for Ba^{2+} as well as for Ca^{2+} , and the highest peak for Co^{2+} at about the same position as that of Mg^{2+} .

Interestingly, this metal position is surrounded by four acidic residues, suggesting electrostatic interaction between these cations and the negative charges of the carboxyl groups. The distances between the Mg^{2+} position in the derivative (standard deviation of position: 0.16 Å) and carboxyl oxygens in the refined metal-free structure were measured to be roughly 2.0 Å for Asp 10, 4.2 Å for Glu 48, 4.5 Å for Asp 70 and 4.9 Å for Asp 134. Some chemical groups, which possibly coordinate to Mg^{2+} , were found around the position, for instance, the carboxyl oxygens of Asp 10 (2.0 Å) and the carbonyl oxygen of Gly 11 (2.3 Å). In the case of Ba^{2+} , two water oxygens (3.4 Å) additionally lie in a possible range of coordination. The Mg^{2+} position was found to be shifted further inward within the metal binding cavity than those of Ba^{2+} or Ca^{2+} . We observed a systematic shift of ionic centres in a reasonable order, according to the ionic radii of Ba^{2+} , Ca^{2+} , Mg^{2+} and Co^{2+} .

The additional chemical groups for coordination would presumably be derived from a polynucleotide main chain during hydrolysis. In the absence of nucleic acid, however, a basic region comprising Lys 86, Lys 87 and Arg 88, which belong to

an adjacent molecule, is approaching this empty Mg^{2+} -binding site by electrostatic interaction with the cluster of acidic residues.

We determined the three-dimensional structures of RNase H mutants whose Glu 48 and Asp 70 were replaced by Gln and Asn respectively K.K., I. Tanaka, S.K., M.I. and K.M., unpublished results). Their structures showed movements of some side chains and formation of extra hydrogen bonds around the Mg^{2+} site which seem to prevent the binding of Mg^{2+} . These conformational changes would account for the deprivation of the activity.

Recent measurements of RNase H by 1H and ^{15}N NMR revealed that the chemical shifts of particular residues around the Mg^{2+} -binding site, such as Asp 10, Gly 11, Asp 70 and Gln 72, were affected by addition of 50 mM Mg^{2+} (Y. Oda and H. Nakamura, unpublished results), supporting the correct identification of the Mg^{2+} -binding site.

The proposed location of Mg^{2+} is consistent with the results of a series of site-directed mutagenesis experiments in which amino-acid residues at various positions were replaced with others (data not shown). The enzymatic activity was almost completely lost by replacement of Asp 10 by Ala or Asn, Glu 48 by Ala or Gln and Asp 70 by Ala or Asn, but not by the replacement of Asp 134 by Asn which is the most distant from Mg^{2+} (4.9 Å). The loss of activity can be explained by disruption of the Mg^{2+} -binding site, although some of the three acidic residues possibly participate directly in hydrolysis of RNA.

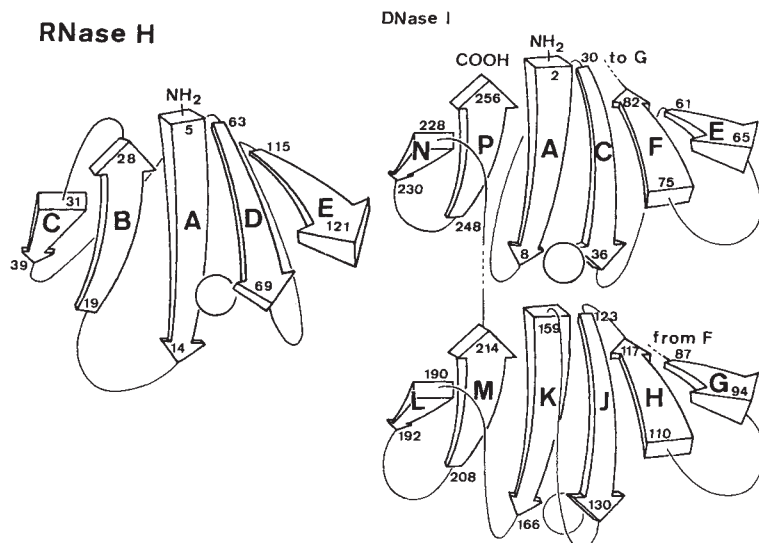


FIG. 3 Schematic representation comparing the directional relationship of β strand topologies between *E. coli* RNase H and DNase I. Circles indicate the Mg^{2+} position for RNase H and the Ca^{2+} position for DNase I, respectively. DNase I (ref. 6) has two β sheets related by an approximate two fold axis but a single catalytic Ca^{2+} , and hence two circles indicate the identical position. The directions and the twist of four β strands (CBAD) in RNase H are identical to those of two β sheets of DNase I (NPAC and LMKJ). In the assumed complex, a DNA-RNA hybrid lies at the end of the β sheet and its axis is roughly normal to the plane. DNase I was reported to bind a double helical DNA in a similar manner^{4,5}.

The four acidic residues around the Mg^{2+} -binding site, Asp 10, Glu 48, Asp 70 and Asp 134, have recently been reported to be completely conserved in the alignment of 26 different assorted sequences from retroviruses, retrovirus-like entities and RNase H from *E. coli*². This conservation can now be explained by the preservation of the Mg^{2+} -binding site, which is essential for activity.

RNase H does not contain the DNA-binding motif consisting of helix-turn-helix, which can commonly be found in repressors¹⁶. The minor domain, α III and the following loop, contains many basic residues (Fig. 1a) whose side chains mostly point into the solvent region. This feature implies that the minor domain is involved in binding to a DNA-RNA hybrid. It is reasonable to assume that a straight DNA-RNA hybrid double helix would bind to the enzyme, along the line linking the acidic Mg^{2+} -binding site with the basic minor domain. A molecular graphics display of the electrostatic potentials¹⁷ on the solvent-accessible surface indicates the cluster of negative charges around the Mg^{2+} -binding site and the minor domain covered with positive charges (Fig. 2).

We could recognize no significant similarity to the three-dimensional structures of nucleases such as RNase A¹⁸⁻¹⁹, RNase T₁²⁰⁻²¹, barnase²² or staphylococcal nuclease²³. With respect to specificity and substrate preference, RNase H seems to be similar to pancreatic DNase I. Both enzymes act on double-stranded polynucleotides without much specificity for base sequence, and both also require metal cations for catalytic reactions (Ca^{2+} for DNase I).

The similarity emerges from the comparison of the β sheets between RNase H and DNase I³⁻⁶ (Fig. 3). The Mg^{2+} -binding site is located near the amino end of β B and the carboxyl ends of β A and β D. Suck and colleagues reported the identification of the amino-acid residues involved in the catalytic reaction and DNA recognition in DNase I⁵⁻⁶. The catalytic Ca^{2+} -binding site is located at the amino end of β P, at the carboxy ends of β A and β C in the first β sheet, and at the amino end of β M, as well as at the carboxyl ends of β K and β J in the second β sheet. Interestingly, the directional relationships of β C to β D

and the twist of β sheet in RNase H are the same as those of the corresponding β strands in two β sheets of DNase I. In both enzymes, the numbers of residues forming β strands range mostly from 7 to 10.

Note added in proof: We are still continuing the crystallographic refinement, extending the intensity data to 1.48 Å resolution. The current *R*-factor is 20.9% on all data from 5.0 to 1.48 Å, and the electron density map has been considerably improved.

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Advances in automated DNA sequencing

R. Cathcart

Sequencing DNA is a labour intensive and expensive process. At an estimated cost of \$3–5 (US) per base pair, it would require about \$25 million (US) to complete the sequence of *Escherichia coli*¹.

THIS article looks at the progress in automated DNA sequencing to increase throughput and reliability. New hardware and improvements in sequencing chemistry developed by Applied Biosystems (ABI) are discussed.

Hardware developments

The ABI Model 373A automated DNA sequencing system can run 24 templates simultaneously. One of the rate-limiting steps now becomes performing the sequencing reactions. The risk of pipetting errors and sample mix-ups can increase with this number of samples. A robotic workstation is under development at ABI that is designed to perform sequencing reactions on up to 24 different templates simultaneously (see Fig. 1).

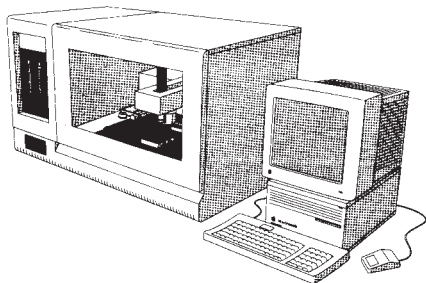


FIG. 1 DNA sequencing robotic workstation.

The worksurface contains refrigerated areas for storing labile reagents such as enzymes and templates, as well as an area for ambient storage. Reagents are pipetted from their storage area to the enzyme reaction module (ERM) using a syringe-based system coupled to a fixed stainless steel probe tip at the end of the robotic arm (see Fig. 2).

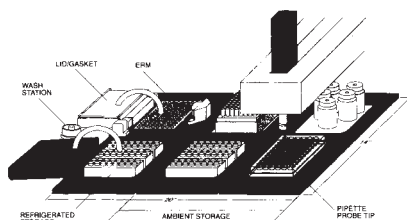


FIG. 2 Details of the worksurface.

An important design criterion of the ERM is evaporation control. This requirement is dictated by the high temperature (95 °C) and small volumes (5 µl) of *Taq* DNA polymerase-based

sequencing protocols. An oil 'cap' is effective in preventing evaporation in manual methods, but is not suitable in automated systems. Instead, the ERM has a lid that automatically closes to seal the enzyme reactions. Wells are machined into an aluminium plate, which is then coated with an inert polymer. The well size is matched to the volume of the reaction to minimize condensation. A gasket in the lid seals all 96 wells by compressing on a machined rim. To prevent condensation from occurring on the lid while in the closed position, it is heated to 2 °C above the reaction temperature.

The aluminium plate is a thermal cycler with a temperature range from 2 to 95 °C. The high thermal conductivity of the aluminium ensures rapid and uniform heating or cooling of the samples. To initiate all reactions simultaneously, the robot loads the ERM at 4 °C and then rapidly heats the plate to the desired temperature. This design prevents evaporation and ensures temperature uniformity between wells. The obvious disadvantage of a reusable plate is that it must be cleaned. However, a relatively simple and rapid method for purging the block between reactions has been determined.

Reagents are pipetted using a single stainless steel probe tip coupled to the syringe pumps. A positive displacement effect is achieved by filling the pumps and line with water. The system can detect when the probe tip touches a liquid surface because the stainless steel probe tip has capacitive sensing. This allows reagents to be delivered at the surface of the reaction, which minimizes probe tip contamination. Increased reliability and lower cost are also achieved with this design because disposable tips do not have to be coupled and uncoupled from the system.

The most important design criterion, however, is the ability to pipette sub-microlitre quantities. Prototype instruments are able to pipette 1 µl and 0.1 µl with a variation of one and eight per cent, respectively. This level of performance was impossible with disposable tip systems. Again, the disadvantage is the probe tip must be washed between pipetting steps.

Prototype instruments are able to perform fluorescent primer sequencing reactions using T7 or *Taq* DNA

polymerase on 24 samples in less than 4.5 hours. Less than one hour is required to clean the ERM between runs. One robot can easily supply the needs of two automated sequencers, such as ABI's 373A.

With sequencing protocols, contamination between samples is not a problem. The normal ERM and probe tip washing protocols are effective at cleaning the system. The obvious concern is for polymerase chain reaction (PCR) applications. Recent experiments suggest that both the wells and the probe tip can be cleaned of amplified DNA by use of dilute nitric acid. Further work is needed in this area, but it seems likely that a cleaning protocol suitable for PCR applications can be devised.

Chemistry improvements

Although the robotic workstation reduces labour requirements and error, and increases the reliability of sequencing, not all applications require this level of throughput. Improvements in the *Taq* sequencing protocol have reduced both the labour requirement and cost of performing sequencing reactions manually. This has been achieved with linear amplification of signal by thermal cycling with a single fluorescent primer (in contrast to the exponential amplification achieved with PCR). Both labour and error are reduced for manual sequencing because the separate annealing step is no longer needed and a denaturation step is not required with double-stranded templates. Costs are reduced because less enzyme and template are required. The need for less template permits the use of larger inserts for primer-directed sequencing, such as cosmid sequencing in which the mole per cent of real target is considerably less than with M13 and plasmids (R. Kaiser, personal communication). Fluorescent signal level remains high because of the linear amplification. The combination of the robot and the *Taq* cycle sequencing protocol should allow further reductions in the amounts of reagents required. □

Richard Cathcart is DNA Sequencing Group Leader at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA. For more information, fill in reader service number 100.

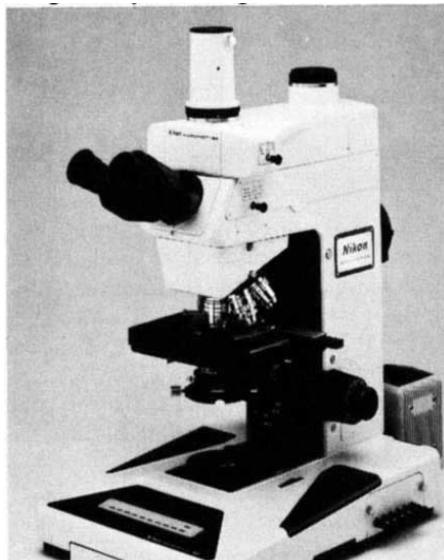
1. Watson, J.D. *Science* **248**, 44–49 (1990).

Lab Week product profile

New lines in laboratory hardware, including a video image recorder and a low-volume evaporator, will be on display at next week's British Laboratory Week exhibition to be held in London.

K.W. KIRK will be displaying an **epi-fluorescence illumination unit** for field and laboratory use or on-the-spot diagnoses on stand D1446 at British Lab Week (Reader Service No. 101). The compact, modified standard McArthur microscope measures $7.6 \times 17.8 \times 15.2$ cm and uses epi-illumination of the specimen to provide a high level of illumination while protecting the user from the harmful effects of UV, says Kirk. The £2,980 (UK) McArthur fluorescence equipment provides magnifications of $100\times$, $400\times$ and $1,000\times$ and is useful where another fluorescence microscope system would either be too costly or impractical.

The Microphot-SA from Nikon is a **microscope designed for image analysis** applications where CCTV observations and photometry are more important to the researcher than photomicrography alone (Reader Service No. 102). The microscope is equipped with two output ports: one for a CCTV camera and the other for photomicrography. The permanent CCTV image analysis configuration is achieved



Microphot-SA: for a permanent CCTV image analysis set-up.

by the use of two independent three-way beam splitters, which provide all possible combinations of observation, CCTV and photography. The Microphot-SA can be linked to a PC via a RS232 interface for either remote control or recording purposes. Key automatic features of the microscope include auto-focus, a motorised nosepiece and condenser, and pre-centred lamphouse. See Nikon on stand K1202.

The FreezeFrame **video image recorder** from Polaroid can prepare 35-mm colour or black and white slides and prints from screen images (Reader Service No. 103). FreezeFrame is PAL-compatible and accepts RGB analogue and RGB/TTL computer input signals. Consequently,



FreezeFrame for slide and print production from video images.

images can be taken from a VCR, VTR, laser disk player or straight from a video camera and recorded on instant photographic prints or 35-mm slides for use in lectures, file records or reports. The instrument 'grabs' a video field from the 'live' image, which can then be adjusted for colour, contrast and brightness before it is captured on film. When used with Polaroid's instant film power processor, FreezeFrame can perform on-the-spot processing of Polaroid's 35-mm full tonal range black and white PolaPan slides, normal-contrast PolaChrome or high-contrast PolaChrome colour slides (recommended for computer image recording). FreezeFrame can also record images on Polaroid's AutoFilm. The motorised Polaroid 'back', which is powered by the host equipment, can produce self-developing instant black and white or colour hard-copy prints in a 3×4 -inch format. Polaroid's products will be on show on stand K1200.

Focusing on electrophoresis

Fotodyne has a new **no-tape electrophoresis chamber** — the Model 1214 — for running 12×14 -cm agarose gels or half-lengths, if required (Reader Service



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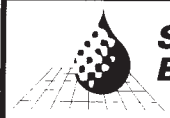
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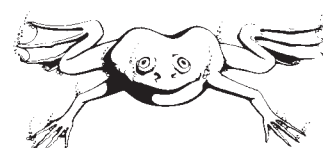
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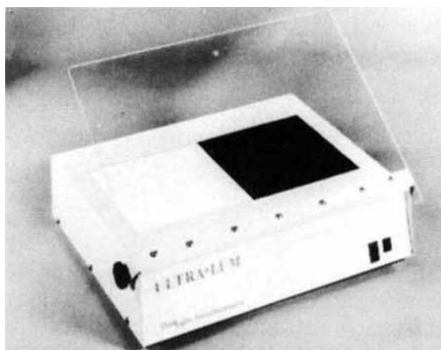
No. 104). The Model 1214's UV-transparent tray is designed to allow gel casting to be performed in the chamber, with no need for taping. By placing the tray sideways in the chamber, end gaskets create a tight, leak-proof seal for gel formation, says Fotodyne. After the gel has set, the tray is then returned to its normal position. The electrophoresis chamber is supplied with partially shielded platinum electrodes, 12- and 20-tooth combs, power leads attached to the lid, buffer exchange ports, and a buffer capacity of 500 ml. The chamber's design also features an evaporation gap to minimize the problem of condensation within the unit, and non-skid rubber feet to provide stability. The Model 1214 costs \$225 (US).

The Chef Mapper **pulsed field gel electrophoresis system** from Bio-Rad sets out to eliminate some of the trial and error associated with optimizing pulsed field separations (*Reader Service No. 105*). Parameters for switch time, pulse angle, switch time ramp, voltage, run time, agarose type and concentration, buffer type and concentration, and temperature are set automatically on the basis of stored protocol information and entered details of DNA size. More experienced users can bypass the algorithm that controls the automation process. For example, changing the pulse angle from 120° to 96° can, says Bio-Rad, cut run times for megabase DNAs in half. Other key features of the Chef Mapper system include multi-state programming of up to 15 vectors per pulse cycle, linear and non-linear switch time ramps, and secondary pulses. H136 is the Bio-Rad stand.

The **megabase restriction mapping system**, which comes in kit form from New England BioLabs, contains all the reagents necessary to resolve a wide range of restriction fragments by pulsed field gel electrophoresis of chromosomal DNA (*Reader Service No. 106*). Each \$125 (US) kit contains restriction endonuclease for digestion of agarose-embedded chromosomal DNA, 10× reaction buffer, control bacterial chromosomal DNA substrate, and lambda ladder and yeast chromosome pulsed field gel markers. Also included in the kit is the GelSyringe system for dispensing agarose plugs of consistent size and quality, says NEB.

Molecular marketplace

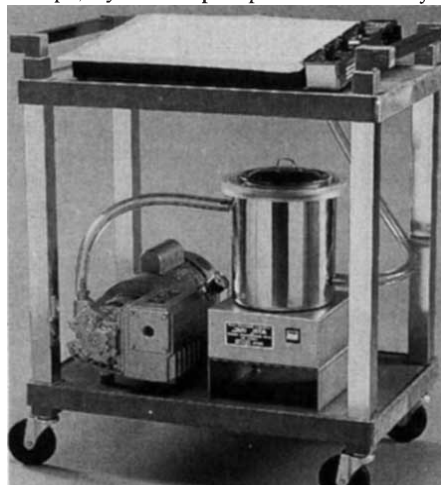
The Ultra-Lum **dual-light transilluminator**, distributed by VWR Scientific, features two light sources in one instrument: a UV light source and a 'cool' fluorescently lit working surface (*Reader Service No. 107*). Two models are available: the \$1,295 (US) UVBW-20, which features four 8-W, 300-nm UV tubes and provides the sensitivity required for viewing ethidium bromide-stained gels while



Ultra-Lum dual-light transilluminator features a fluorescently lit workstation.

preventing photodamage, photobleaching, photoniccking and photodimerization, says VWR; and the \$1,095 (US) UVAW-20, which has four 8-W, 365-nm UV tubes. Both transilluminators also feature two 8-W cool-white fluorescent tubes for viewing silver- and Coomassie blue-stained protein gels and methylene blue-stained nucleic acid gels, autoradiographs and microtitre plates.

Key features of the new GDS-1 **gel drying system** from ATR/AHL are large gel dryer slab capacity, variable temperature control, acid-resistant design, and an automatic vent/drain mechanism (*Reader Service No. 108*). The \$2,725 (US) system comes complete with gel dryer, pump, automated dry ice trap, vacuum tubing, clamps, synthetic pump oil and trolley.



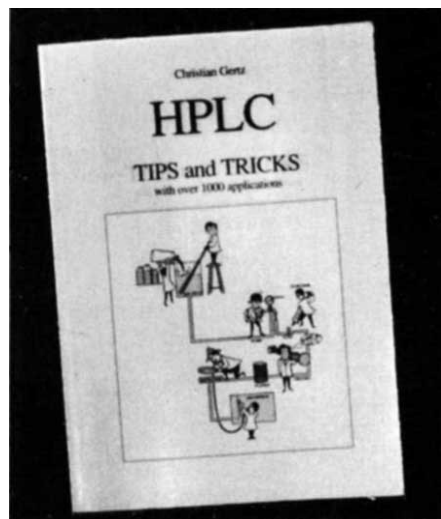
Complete gel drying system on wheels.

The 36 × 44-cm drying surface can accommodate six 14 × 16-cm slab gels or three 14 × 32-cm slabs. Gel drying temperatures can be adjusted over the range of 40 °C to 80 °C. The dryer features two timers: one to control the vacuum pump, the other to operate the heating circuit. Both dry heat and reduced pressure are used to dehydrate slab gels and bond them permanently either to filter paper or transparent porous cellophane. The automated vacuum condensing trap is designed to both protect the vacuum pump in corrosive environments and offer a means of collecting and discarding unwanted solvents.

The **miniblotter incubation system** from Biometra is designed to provide a rapid method for screening antibodies on western blots by eliminating the need to cut blot membranes (*Reader Service No. 109*). Up to 45 antibodies can be screened on a single blot, without problems of cross-channel leakage, says Biometra. The screening procedure requires only small volumes of antibody solution — typically 50–250 µl. The £495 (UK) miniblotter incubation system has a detachable manifold to allow thorough washing of the blot.

Chromatography corner

Tips and Tricks, a new **HPLC textbook** from LDC Analytical, covers all aspects of HPLC practice and should be of interest to novices and experts alike (*Reader Service No. 110*). This 500-page publication covers the theory of chromatographic separation and contains practical advice



HPLC — the theory and the practice.

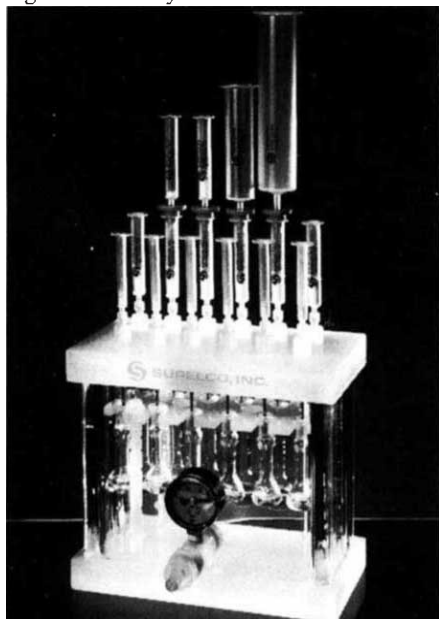
on how to select the most suitable stationary and mobile phase, how to check the reproducibility of your chromatographic system, how to build, operate and maintain an HPLC system, and how to evaluate and quantitate results. The £35 (UK) *Tips and Tricks* book contains 1,000 up-to-date references and will be available on the LDC stand, number K1016.

Polymer Laboratories has brought out a new matched series of PL-GFC **macroporous polymeric resins** with a hydrophilic surface for gel filtration chromatography (*Reader Service No. 111*). The resins can be used for the analysis of a variety of water-soluble macromolecules, including charged and neutral polysaccharides. PL-GFC resins are available in three pore sizes — 300 Å, 1,000 Å and 4,000 Å — for use individually or, if increased resolution or resolution over a broader molecular size range is required, in multi-column sets. The 300 Å and 4,000 Å materials have matched pore sizes to allow them to be used in series to cover polysaccharide molecular weights ranging from several

hundred to about 20 million, says PL. The hydrophilic homogeneous surface and chemical stability of the polymer matrix permits the use of a wide variety of aqueous eluents; this reduces non-specific interactions and enables polysaccharides to be analysed in the presence of proteins, without the problem of irreversible column fouling. See PL Separation Sciences on stand K1328.

Spectra-Physics has introduced the SP8880B **biocompatible autosampler** which is designed for applications where wetted parts made of metal would interfere with metal-catalysed reactions in the sample (*Reader Service No. 112*). All the wetted parts in the SP8880B are made of PEEK (polyetheretherketone) instead of titanium. The company has also replaced the Vespel rotor seal in the rheodyne 7010 valve with one made of Tefzel, which tolerates a wider pH range, says Spectra-Physics. The autosampler holds up to 80 samples and can perform ten injections per sample. When the SP8880B is teamed up with an inert pump and a detector that has a biocompatible flow cell, users can configure a complete biocompatible LC system that can also be integrated into Spectra-Physics' communications network — Labnet. Prices start at \$17,000 (US).

Using the Supelco Visiprep **solid-phase extraction vacuum manifold**, it is possible to clean up to 12 samples simultaneously for HPLC or other analyses (*Reader Service No. 113*). The apparatus has twelve independently-controlled screw-type valves built into the cover, which provide precise flow control through each SPE tube. The vacuum is adjusted using the vacuum bleed valve and gauge. Such control ensures, says Supelco, that the packing does not dry out in some tubes while



Supelco's 12-sample vacuum manifold.

others are still draining. The £540 (UK) SPE vacuum manifold has a rugged, clear-glass basin that provides clear visibility during the extraction process and will not fog or discolour even when exposed to solvents such as hydrochloric acid and tetrahydrofuran. The polypropylene collection-vessel rack holds autosampler and small scintillation vials, 10- and 16-mm test tubes, and 1-, 2-, 5- and 10-ml volumetric flasks. By adding an optional plate, samples can be collected in up to ten large (20-ml) scintillation vials. The stainless steel guide needles can easily be removed for cleaning or replacement. Alternatively, where sample-to-metal contact must be avoided, Supelco can provide Teflon solvent guide needles.

Tools of the trade

The new combination **hotplate/stirrer** from Fisher Scientific uses infrared light to provide temperatures of up to 615 °C (*Reader Service No. 114*). The hotplate has a rapid thermal response time and will boil one litre of water in nine minutes, says Fisher. Two models are available: the smaller \$795 (US) Model 4100 with a six-inch diameter heating circle (28 in²) and the larger \$850 (US) eight-inch Model 6100 version (50 in²). Stirring speeds can be set to between 60 and 1,500 r.p.m. Infrared produced by the molybdenum disilicide element housed beneath the black, glass ceramic top plate glows orange when the hotplate is in use. For safety purposes, the hotplate has a stainless steel lip for catching spills.

Neslab Instruments, exhibiting on stand C512, has introduced a new -80 °C **personal freezer** — the PF8-80 — for the safe and secure storage of radioisotopes and biological samples (*Reader Service No. 115*). Both the -80 °C model and the earlier -40 °C model are fitted with an audio/visual alarm. There is also provision for a remote alarm and temperature monitoring. High security is the freezer's key feature, which is achieved by placing the set point and alarm controls inside the door jamb. Other features include a lockable door and large LED. Neslab's freezer sells for £2,100 (UK).

TurboVap LV, the automated **low-volume evaporator** from Zymark, is designed to evaporate to dryness 1–50 samples simultaneously with volumes from 1–30 ml (*Reader Service No. 116*). The \$4,900 (US) evaporator uses nitrogen gas to create a vortex action in the sample and can, says Zymark, evaporate 10 ml of MeCl₃ in less than 15 minutes at 38 °C or 10 ml hexane in less than nine minutes at 52 °C. The compact, TurboVap LV uses standard 16 × 100-mm or 20 × 150-mm tubes. The temperature of the bath can be varied from between ambient and 90 °C. Interfacing the TurboVap LV to Zymark's

BenchMate workstation provides automated sample preparation and evaporation. See stand A448.

On show for the first time at British Lab Week on Grant Instrument's stand, number H150, will be a **portable incubator** that provides a useful alternative to conventional CO₂ incubators (*Reader Service No. 117*). Because of the incubator's portable design, samples can be kept to hand while the user is working at the bench where space is often limited. The



Portable incubator — see stand H150.

incubator can provide a stable temperature of either 37 °C or 56 °C and an internal atmosphere of constant humidity and five per cent CO₂ in air. After initial gassing with a bottled gas mixture, conditions are maintained for up to 48 hours, says Grant. The incubator can accommodate test tubes, petri dishes or a combination of both. It can also be converted to a standard heating block or stand-alone base plate.

Quantitative analysis

Pharmacia LKB Biochrom will use Lab Week to launch a new Ultrospec III **UV/visible spectrophotometer** with Microsoft Windows PC-based applications software — see stand G182 (*Reader Service No. 118*). Ultrospec III has a spacious sample



A Lab Week launch for Pharmacia's UV/visible spec and colour software.

compartment and can be equipped with a wide range of accessories, including the Autofill sipper rapid sampler, thermostatted cell holders and funnel flow cells, all of which allow the instrument to be adapted to suit laboratory needs. The new spectro colour software, operating in a Microsoft Windows environment with mouse control if possible, can be used with IBM PCs or compatibles. The software has three modules: enzyme kinetics for enzyme

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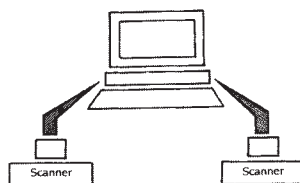
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Reader Service No.49

catalysing reaction studies, wavescan for wavelength scanning, and standard curve for quantitative and colorimetric analyses. Pharmacia's 20-page product brochure describes the spectrophotometer in detail.

Molecular Dynamics now offers researchers a choice of three **computing densitometers** (Reader Service No. 119). Two new instruments — the Model 300E and 300S — have been added to the product line to provide faster data analysis for gels, films and membranes. The Model 300S, which is fitted with a 16 MHz Intel 80386SX microprocessor, can display and quantitate image data up to 60 per cent faster than the 300A, says Molecular Dynamics. All three models are designed to scan electrophoresis gels, radiographic films and blotting membranes as large as 36 x 42 cm in less than three minutes. All three use the same helium-neon laser, light-collection cylinder and Image-Quant software, which eases the user through the process of data collection and analysis. See stand G629.

ScanDuet, the automated TLC **radioimaging and analysis system** from Bioscan is designed for high-throughput TLC applications (Reader Service No. 120). The instrument, which consists of two automated scanners linked up to a multi-tasking computer, can load and read up to eight 20 x 20-cm plates without user intervention. Users have a choice of 1- or 2-D detection. ScanDuet's detectors can measure 50 d.p.m. of ³H or ¹⁴C in ten minutes, says Bioscan. With low-activity



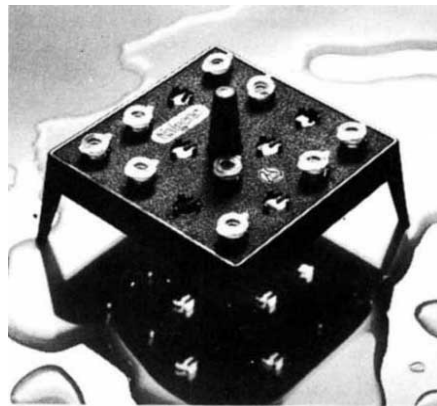
High throughput TLC image analysis.

samples, 2-D images can be produced in a day and not weeks as with autoradiography. ScanDuet's windowless detector and the use of uncovered plates contribute to the instrument's high sensitivity. To suit user needs, both lane width and resolution can be altered by changing snap-on collimators. The TLC-specific software that accompanies ScanDuet allows the user to display and quantitate data in 1-, 2- and 3-D. Features of the software include automatic peak search, a fine-tuning capability for regions of interest, and computa-

tion of peak location, total counts, c.p.m. and per cent of total activity.

Lab consumables

Throw away your polystyrene tube holders, Nalge is offering a **floating microtube rack** for 0.5-ml Eppendorf tubes (Reader Service No. 121). The rack holds sixteen 0.5-ml tubes in a 4 x 4 array, allowing incubation of samples at elevated or reduced temperatures in water or ice baths. Made of polypropylene, the rack offers, says Nalge, temperature resistance from -70 °C to 100 °C. The rack



Nalge's floating microtube rack.

comes with moulded-in handles to assist removal from a water bath. Nalge is on stand K1074.

Hepa-Vent, the lightweight **in-line microfiltration device** from Whatman can be used in sterile air or gas venting applications (Reader Service No. 122). Each filtration device features a polypropylene housing with stepped hose barbed connections and a glass microfibre filter medium that is laminated on both sides and slightly hydrophobic. The device, which is autoclavable, is designed to retain 99.97 per cent of airborne particles that are ≥3 µm. Hepa-Vent's disc format provides 16 cm² of filtration area: for larger air flows, however, Hepa-Vent is available with a filtration area of 540 or 1,260 cm². See stand G164.

Tissue Culture Services, exhibiting on stand L1192, is offering EquiCell **donor equine serum** as an alternative to fetal calf serum (Reader Service No. 123). EquiCell is produced from a controlled herd of donor horses that are screened by BioResearch — Ireland's National Cell and Tissue Culture Centre in Dublin. The serum is suitable for the growth of hybridomas and myelomas, epithelial cell lines, fibroblasts and recombinant cell lines such as CHO and BHK. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.